



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C07K 14/575, 14/78, A61K 38/22, 38/39, G01N 33/68	A1	(11) International Publication Number: WO 97/38014 (43) International Publication Date: 16 October 1997 (16.10.97)
(21) International Application Number: PCT/US97/06280 (22) International Filing Date: 3 April 1997 (03.04.97) (30) Priority Data: 08/627,636 4 April 1996 (04.04.96) US (71) Applicant: AMGEN INC. [US/US]; Amgen Center, 1840 De Havilland Drive, Thousand Oaks, CA 91320-1789 (US). (72) Inventor: BENNETT, Larry, G.; 2977 Sunflower Street, Thousand Oaks, CA 91360 (US). (74) Agents: ODRE, Steven, M. et al.; Amgen, Inc., Amgen Center, 1840 De Havilland Drive, Thousand Oaks, CA 91320-1789 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
(54) Title: FIBULIN PHARMACEUTICAL COMPOSITIONS AND RELATED METHODS (57) Abstract The present invention relates to fibulin protein, fibulin/OB protein complex and pharmaceutical compositions thereof. In addition, the present invention also relates to methods of making and using the above proteins and compositions.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon	KR	Republic of Korea	PL	Poland		
CN	China	KZ	Kazakhstan	PT	Portugal		
CU	Cuba	LC	Saint Lucia	RO	Romania		
CZ	Czech Republic	LI	Liechtenstein	RU	Russian Federation		
DE	Germany	LK	Sri Lanka	SD	Sudan		
DK	Denmark	LR	Liberia	SE	Sweden		
EE	Estonia			SG	Singapore		

FIBULIN PHARMACEUTICAL COMPOSITIONS AND RELATED METHODS

FIELD OF THE INVENTION

5

The present invention relates to fibulin protein, fibulin/OB protein complex and pharmaceutical compositions thereof. In addition, the present invention also relates to methods of making and using the above proteins and compositions.

BACKGROUND

Although the molecular basis for obesity is largely unknown, the identification of the "OB gene" and protein encoded ("OB protein") has shed some light on mechanisms the body uses to regulate body fat deposition. Zhang et al., Nature 372: 425-432 (1994); see also, the Correction at Nature 374: 479 (1995). The OB protein is active in vivo in both ob/ob mutant mice (mice obese due to a defect in the production of the OB gene product) as well as in normal, wild type mice. The biological activity manifests itself in, among other things, weight loss. See generally, Barinaga, "Obese" Protein Slims Mice, Science 269: 475-476 (1995). It is known, for instance, that in ob/ob mutant mice, administration of OB protein results in a decrease in serum insulin levels, and serum glucose levels. It is also known that administration of OB protein results in a decrease in body fat. This was observed in both ob/ob mutant mice, as well as non-obese normal mice. Pellemounter et al., Science 269: 540-543 (1995); Halaas et al., Science 269: 543-546 (1995). See also,

Campfield et al., Science 269: 546-549 (1995) (Peripheral and central administration of microgram doses of OB protein reduced food intake and body weight of ob/ob and diet-induced obese mice but not in db/db obese mice.)

- 5 In none of these reports have toxicities been observed, even at the highest doses.

The characterization of OB protein in blood has not been accomplished to any significant degree. For example, it is not known whether OB protein
10 circulates complexed to other substances in blood, and, if so, what effects these other substances may have. Such binding complexes or proteins may, for example, act as antagonist, or may have a protective effect, and contribute to the stability of either exogenously
15 administered or naturally occurring OB protein present in the body.

As fully described below, the present invention relates to the association of fibulin with OB protein. The fibulins are known to be plasma proteins.
20 See Tran et al., J. Biol. Chem. 270: 19458-19464 (1990). There are two known types of fibulins, type 1 and type 2. Agraves et al., J. Cell Biol. 111: 3155-3164 (1990). Of fibulin type 1, there are 4 variants or isoforms, denoted in the literature as "A", "B", "C",
25 and "D". Id. at Figure 6. Generally, fibulin type 1 "A" is a 537 amino acid protein. Argraves, et al., Cell 58:623-629(1989). Variants "B", "C" and "D" have conserved amino acids 1-537, but have altered C-termini: "B" has amino acids 538-572, "C" has amino acids 538-
30 654; and "D" has amino acids 538-674. The polypeptides predicted from the nucleotide sequences of the CDNA's have molecular masses of 58,670, 62,561, 71,551 and 77,274 for fibulin type 1 variants A-D respectively. See Argraves et al., supra. These forms are also rich
35 in cysteine, approximately 11 mole %. Id. at 3160.

Fibulin type 1 is known to co-purify with fibrinogen, and to bind to various extracellular matrix proteins such as fibronectin, laminin, and nidogen. See Tran et al., supra. Fibulin type 1 has been observed in
5 the blood in concentrations of 30-40 µg/ml, id., and is thought to be, among other things, a structural protein that contributes to the elastic properties of connective tissue, or is involved in the process of fibrogenesis. Roark et al., J. Histochem. Cytochem. 43: 401-411
10 (1995). Fibulin protein, the DNA encoding it, as well as antibodies reactive with fibulin, have also been characterized by Argraves et al. in the PCT published application No. WO 91/02755.

As for fibulin type 2, it is larger than
15 fibulin type 1 and possesses a 408 amino acid N-terminal domain not found in fibulin type 1. See Zhang et al., Genomics 22: 425-430 (1994). Fibulin type 2 has been observed in the skin, including the epidermal layer, the dermis and adipose tissue, as well as the keratinocyte
20 layer of the hair, the epithelial layer of the cornea, the aortic intima, and around small vessels in the kidney and liver. Id. at 428. In addition, serum levels of fibulin type 2 have been noted to be 1000 fold lower than fibulin type 1. Id. at 1269.

25 It is desirable to have a pharmaceutical composition which enhances the effectiveness of either exogenously administered or endogenous OB protein. Such compositions would be useful for example, to reduce or eliminate the need for exogenous OB protein
30 administration.

SUMMARY OF THE INVENTION

The present invention stems from the observation that fibulin type 1 binds to OB protein.

5 Such binding is thought to have a potentiating effect of OB protein. While not wishing to be bound by theory, such potentiating effect is thought to be related to fibulin's protection of OB protein in circulation. Such protection may increase the stability of OB protein in
10 the blood, and therefore permit biological activity enhanced relative to OB protein alone. Accordingly, fibulin can be used as a pharmaceutical composition to enhance the effectiveness of either exogenously administered or endogenous OB protein.

15 Therefore, one aspect of the present invention is a pharmaceutical composition containing fibulin type 1 protein. This composition can contain either fibulin type 1 variant "A" (amino acids 1-537), fibulin type 1 variant "B" (amino acids 1-572), fibulin type 1 variant
20 "C" (amino acids 1-654), fibulin type 1 variant "D" (amino acids 1-674) or a combination of the above. In addition, the pharmaceutical composition can also contain the methionyl form of any of the above fibulin type 1 variants having a methionyl residue at the
25 N-terminus (at the -1 position). Other analogs, amino acid substitutions, derivatives, truncated versions of fibulin type 1 variants and combinations thereof are also within the scope of this invention. This would include fibulin type 2 as well since type 2 is the same
30 as type 1 with an additional 408 amino acid N-terminal domain added. Furthermore, the fibulin can also be in a dimer form. The pharmaceutical composition includes a pharmaceutically acceptable carrier, diluent, adjuvant, solubilizer and/or stabilizer.

A second aspect of the present invention includes OB protein complexed to the fibulin type 1 protein whether variant A, B, C, D or a combination thereof is used. The OB protein can be chemically, 5 covalently, or ionically attached or complexed to fibulin type 1, as well as, any other means of association. In addition, such binding can occur in the presence of divalent cations, especially calcium, (Ca^{++}). Furthermore, the N-terminal methionyl form of 10 fibulin type 1 can also be used. In forming the OB protein/fibulin type 1 complex, various forms of the OB protein, analogs or derivations thereof can be used as well as various forms of fibulin type 1, analogs, derivatives or combinations thereof.

15 A third aspect of the present invention provides a pharmaceutical composition containing the OB protein/fibulin type 1 complex as described above in a pharmaceutically acceptable carrier, diluent, adjuvant solubilizer and/or stabilizer.

20 A fourth aspect of the present invention includes a method of treating obesity, hyperlypedemia, Type II diabetes and other obesity related disorders by administering an effective amount of one or more of the pharmaceutical compositions containing fibulin type 1 or 25 fibulin type 1/OB protein complex. Such treatment includes use of variants, analogs, derivations or combinations of fibulin type 1 and/or OB protein. Treatment using the above includes, but is not limited to, the reduction of serum lipid levels, cholesterol and 30 triglyceride levels, reduction or prevention of arterial plaque, and the reduction of hypertension and related gall stone formation.

A fifth aspect of the present invention is a method of preparing the pharmaceutical compositions 35 discussed above by admixing purified and isolated

fibulin type 1 or OB protein/fibulin type 1 complex, with a pharmaceutically acceptable carrier, diluent, adjuvant solubilizer and/or stabilizer. This method includes use of any variants, N-terminal methionyl
5 versions, analogs, derivatives or combinations of fibulin type 1 or OB protein/fibulin type 1 complex.

Another aspect of the present invention includes a method for detecting the presence of OB protein in a biological sample. Such a method comprises
10 the steps of incubating the sample with fibulin type 1, or variants, analogs or derivatives as described above, under conditions that allow binding of the fibulin type 1 to OB protein in a sample. The amount of OB protein bound to the fibulin type 1 in the complex is then
15 determined.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 represents the presence of fibulin type 1 binding to OB protein. Lane 1 contains the wash from the control resin. Lane 2, contains the wash from the OB-linked resin. Lanes 3-10 contain various wash fractions and controls. Fibulin is located in Lane 2 at band ~ 85 kD.

25 Figure 2 represents the amino acid and DNA sequences for recombinant murine met-OB protein (SEQ. ID. NOs. 1, 2 and 3).

Figure 3 represents the amino acid and DNA sequences for recombinant human met-OB analog (SEQ. ID.
30 NOs: 4, 5 and 6).

Figure 4 represents the amino acid sequence for human fibulin type 1 isoform or variant A (SEQ. ID. NO. 7).

Figure 5 represents the amino acid sequence for human fibulin type 1 isoform or variant B (SEQ. ID. NO. 8).

Figure 6 represents the amino acid sequence for human fibulin type 1 isoform or variant C (SEQ. ID. NO. 9).

Figure 7 represents the amino acid sequence for human fibulin type 1 isoform or variant D (SEQ. ID. NO. 10).

10

DETAILED DESCRIPTION

Fibulin may be selected from fibulin type 1 isoforms or variants A, B, C or D. In particular, fibulin type 1 may be selected from amino acids 1-537 (variant A) of SEQ. ID. NO. 7 (Figure 4); amino acids 1-572 (variant B) of SEQ. ID. NO. 8 (Figure 5); amino acids 1-654 (variant C) of SEQ. ID. NO. 9 (Figure 6); or amino acids 1-674 (variant D) of SEQ. ID. NO. 10 (Figure 7). As set forth below, the amino acid sequences above can contain a methionyl residue at the -1 position, however, as with any of the present fibulin proteins and analogs, the methionyl residue may be absent. Furthermore, as discussed below, any fibulin protein used can be isolated and purified from naturally occurring sources or recombinantly produced. Such fibulins may also include, incident to expression or otherwise, the leader sequence at position -29 to -1. Such fibulins can also include a dimer form of fibulin.

At a minimum, what is needed is that portion of the fibulin type 1 protein sufficient to complex to the type of OB protein used. This is readily determined by preparing deletion or substitution analogs (for example, using the methods of Alton et al. PCT published application No. WO 83/04053 or other methods available

to those skilled in the art), contacting such prepared portion of fibulin type 1 with the desired OB protein under suitable conditions (e.g., physiological conditions), including but not limited to the presence
5 of divalent cations such as Calcium (Ca^{++}) and ascertaining the presence or amount of fibulin/OB protein complex formed.

One may also use the above amino acid sequences but with various amino acids substituted. For
10 example, one or more cysteine residues may be replaced by an alanine or serine residue and one or more tyrosine residues replaced by a phenylalanine residue.

One may further prepare derivatives, as described below, with chemical moieties connected to the
15 fibulin protein moieties. Such chemical moieties can include water soluble polymers, such as polyethylene glycol or polyamino acid. In addition, the chemical moiety can be attached at solely the N-terminus of the fibulin protein.

20 Hybrid molecules based on the fibulin type 1 variants A, B, C or D can also be prepared. The fibulin type 1 variants have conserved sequences 1-537 but differ from amino acids 538-674. One may substitute another amino acid or add to the existing amino acids
25 one or more of the amino acids from these alternative isoforms to obtain a hybrid molecule. This would include fibulin type 2 which is the same as fibulin type 1 with an additional 408 amino acid N-terminal domain added. Such consensus molecules include but are not limited to
30 the following fibulin protein molecules (using the numbering of SEQ. ID. NOS. 7, 8, 9 and 10):

(a) amino acids 1-537 and (connected to) amino acids 573-654 or amino acids 655-674;

(b) amino acids 1-572 and (connected to)
35 amino acids 655-674;

(c) amino acids 1-537 and (connected to) amino acids 573-654, having one or more of amino acids 538-572 placed between amino acids 537 and 573;

(d) amino acids 1-537 and (connected to)
5 amino acids 655-674, having one or more of amino acids 538-572 and/or 573-654, placed between amino acids 537 and 655; and

(e) amino acids 1-572 and (connected to) amino acids 655-674, having one or more of amino acids
10 573-654 placed between amino acids 572 and 655.

The OB protein may be selected from recombinant murine set forth below (SEQ. ID. NOs. 1, 2 or 3), or recombinant human protein as set forth in Zhang et al., Nature, supra, herein incorporated by
15 reference) or those lacking a glutaminy residue at position 28. (See Zhang et al, Nature, supra, at page 428). One may also use the recombinant human OB protein analog as set forth in SEQ. NOs. 4, 5 or 6 which
20 contains: (1) an arginine in place of lysine at position 35; and (2) a leucine in place of isoleucine at position 74. (A shorthand abbreviation for this analog is the recombinant human R->K³⁵, L->I⁷⁴). The amino acid
sequences for the recombinant human analog and recombinant murine proteins are set forth below with a
25 methionyl residue at the -1 position, however, as with any of the present OB proteins and analogs, the methionyl residue may be absent.

The murine protein is substantially homologous to the human protein, particularly as a mature protein,
30 and; further, particularly at the N-terminus. One may prepare an analog of the recombinant human protein by altering (such as substituting amino acid residues), in the recombinant human sequence, the amino acids which diverge from the murine sequence. Because the
35 recombinant human protein has biological activity in

mice, such analog would likely be active in humans. For example, using a human protein having a lysine at residue 35 and an isoleucine at residue 74 according to the numbering of SEQ. ID. NO. 6, wherein the first amino acid is valine, and the amino acid at position 146 is cysteine, one may substitute with another amino acid one or more of the amino acids at positions 32, 35, 50, 64, 68, 71, 74, 77, 89, 97, 100, 105, 106, 107, 108, 111, 118, 136, 138, 142, and 145. One may select the amino acid at the corresponding position of the murine protein, (SEQ. ID. NO. 3), or another amino acid.

One may further prepare "consensus" molecules based on the rat OB protein sequence. Murakami et al., Biochem. Biophys. Res. Comm. 209: 944-952 (1995) herein incorporated by reference. Rat OB protein differs from human OB protein at the following positions (using the numbering of SEQ. ID. NO. 6): 4, 32, 33, 35, 50, 68, 71, 74, 77, 78, 89, 97, 100, 101, 102, 105, 106, 107, 108, 111, 118, 136, 138 and 145. One may substitute with another amino acid one or more of the amino acids at these divergent positions. The positions in bold print are those which in which the murine OB protein as well as the rat OB protein are divergent from the human OB protein, and thus, are particularly suitable for alteration. At one or more of these positions, one may substitute an amino acid from the corresponding rat OB protein, or another amino acid.

The positions from both rat and murine OB protein which diverge from the mature human OB protein are: 4, 32, 33, 35, 50, 64, 68, 71, 74, 77, 78, 89, 97, 100, 102, 105, 106, 107, 108, 111, 118, 136, 138, 142, and 145. A human OB protein according to SEQ. ID. NO. 6 (with lysine at position 35 and isoleucine at position 74) having one or more of the above amino acids deleted or replaced with another amino acid, such as the amino

acid found in the corresponding rat or murine sequence, may also be effective.

In addition, the amino acids found in rhesus monkey OB protein which diverge from the mature human OB protein are (with identities noted in parentheses in one letter amino acid abbreviation): 8 (S), 35 (R), 48(V), 53(Q), 60(I), 66(I), 67(N), 68(L), 89(L), 100(L), 108(E), 112 (D), and 118 (L). Since the recombinant human OB protein is active in cynomolgus monkeys, a human OB protein according to SEQ. ID. NO. 6 (with lysine at position 35 and isoleucine at position 74) having one or more of the rhesus monkey divergent amino acids replaced with another amino acid, such as the amino acids in parentheses, may be effective. It should be noted that certain rhesus divergent amino acids are also those found in the above murine species (positions 35, 68, 89, 100 and 112). Thus, one may prepare a murine/rhesus/human consensus molecule having (using the numbering of SEQ.ID. NO. 6 having a lysine at position 35 and an isoleucine at position 74) having one or more of the amino acids at positions replaced by another amino acid: 4, 8, 32, 33, 35, 48, 50, 53, 60, 64, 66, 67, 68, 71, 74, 77, 78, 89, 97, 100, 102, 105, 106, 107, 108, 111, 112, 118, 136, 138, 142, and 145.

Other analogs may be prepared by deleting a part of the protein amino acid sequence. For example, the mature protein lacks a leader sequence (-22 to -1). One may prepare the following truncated forms of human OB protein molecules (using the numbering of SEQ. ID. NO. 6):

- (a) amino acids 98-146;
 - (b) amino acids 1-32;
 - (c) amino acids 40-116;
 - (d) amino acids 1-99 and (connected to)
- 112-146; and

(e) amino acids 1-99 and (connected to) 112-146 having one or more of amino acids 100-111 placed between amino acids 99 and 112.

In addition, the truncated forms may also have altered one or more of the amino acids which are divergent (in the rhesus, rat or murine OB protein) from human OB protein. Furthermore, any alterations may be in the form of altered amino acids, such as peptidomimetics or D-amino acids.

10

Derivatives

The present fibulin and/or OB protein (herein the term "protein" is used to include "peptide" and OB or fibulin analogs, such as those recited both supra and infra, unless otherwise indicated) may also be derivatized by the attachment of one or more chemical moieties to the protein moiety. If the present pharmaceutical compositions contain as the active ingredient a complex of fibulin and OB protein, one or both of such proteins may be derivatized. The chemically modified derivatives may be further formulated for intraarterial, intraperitoneal, intramuscular, subcutaneous, intravenous, oral, nasal, pulmonary, topical or other routes of administration. Chemical modification of biologically active proteins has been found to provide additional advantages under certain circumstances, such as increasing the stability and circulation time of the therapeutic protein and decreasing immunogenicity. See U.S. Patent No. 4,179,337, Davis et al., issued December 18, 1979. For a review, see Abuchowski et al., in Enzymes as Drugs. (J.S. Holcerberg and J. Roberts, eds. pp. 367-383 (1981)). A review article describing protein modification and fusion proteins is Francis, Focus on Growth Factors 3: 4-10 (May 1992) (published by

Mediscript, Mountview Court, Friern Barnet Lane, London N20, OLD, UK).

The chemical moieties suitable for derivatization may be selected from among various water soluble polymers. The polymer selected should be water soluble so that the protein to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. Preferably, for therapeutic use of the end-product preparation, the polymer will be pharmaceutically acceptable. One skilled in the art will be able to select the desired polymer based on such considerations as whether the polymer/protein conjugate will be used therapeutically, and if so, the desired dosage, circulation time, resistance to proteolysis, and other considerations. For the present proteins and peptides, the effectiveness of the derivatization may be ascertained by administering the derivative, in the desired form (i.e., by osmotic pump, or, more preferably, by injection or infusion, or, further formulated for oral, pulmonary or nasal delivery, for example), and observing biological effects as described herein.

The water soluble polymer may be selected from the group consisting of, for example, polyethylene glycol, copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random or non-random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols, polystyrenemaleate and polyvinyl alcohol. Polyethylene

glycol propionaldehyde may have advantages in manufacturing due to its stability in water.

Fusion proteins may be prepared by attaching polyaminoacids to the fibulin or OB protein (or analog) moiety. For example, the polyamino acid may be a carrier protein which serves to increase the circulation half life of the protein. For the present therapeutic or cosmetic purposes, such polyamino acid should be those which have do not create neutralizing antigenic response, or other adverse response. Such polyamino acid may be selected from the group consisting of serum album (such as human serum albumin), an antibody or portion thereof (such as an antibody constant region, sometimes called "F_C") or other polyamino acids. As indicated below, the location of attachment of the polyamino acid may be at the N-terminus of the fibulin or OB protein moiety, or other place, and also may be connected by a chemical "linker" moiety to the fibulin or OB protein.

The polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the preferred molecular weight is between about 2 kDa and about 100 kDa (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight) for ease in handling and manufacturing. Other sizes may be used, depending on the desired therapeutic profile (e.g., the duration of sustained release desired, the effects, if any on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of the polyethylene glycol to a therapeutic protein or analog).

The number of polymer molecules so attached may vary, and one skilled in the art will be able to ascertain the effect on function. One may mono-derivatize, or may provide for a di-, tri-, tetra-

or some combination of derivatization, with the same or different chemical moieties (e.g., polymers, such as different weights of polyethylene glycols). The proportion of polymer molecules to protein (or peptide) molecules will vary, as will their concentrations in the reaction mixture. In general, the optimum ratio (in terms of efficiency of reaction in that there is no excess unreacted protein or polymer) will be determined by factors such as the desired degree of derivatization (e.g., mono, di-, tri-, etc.), the molecular weight of the polymer selected, whether the polymer is branched or unbranched, and the reaction conditions.

The chemical moieties should be attached to the protein with consideration of effects on functional or antigenic domains of the protein. There are a number of attachment methods available to those skilled in the art. E.g., EP 0 401 384 herein incorporated by reference (coupling PEG to G-CSF), see also Malik et al., Exp. Hematol. 20: 1028-1035 (1992) (reporting pegylation of GM-CSF using tresyl chloride). For example, polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as, a free amino or carboxyl group. Reactive groups are those to which an activated polyethylene glycol molecule may be bound. The amino acid residues having a free amino group may include lysine residues and the N-terminal amino acid residue. Those having a free carboxyl group may include aspartic acid residues, glutamic acid residues, and the C-terminal amino acid residue. Sulfhydryl groups may also be used as a reactive group for attaching the polyethylene glycol molecule(s). Preferred for therapeutic purposes is attachment at an amino group, such as attachment at the N-terminus or lysine group. Attachment at residues important for

receptor binding should be avoided if receptor binding is desired.

One may specifically desire N-terminally chemically modified protein. Using polyethylene glycol as an illustration of the present compositions, one may select from a variety of polyethylene glycol molecules (by molecular weight, branching, etc.), the proportion of polyethylene glycol molecules to protein molecules in the reaction mix, the type of pegylation reaction to be performed, and the method of obtaining the selected N-terminally pegylated protein. The method of obtaining the N-terminally pegylated preparation (i.e., separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated protein molecules. Selective N-terminal chemical modification may be accomplished by reductive alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl group containing polymer is achieved. For example, one may selectively N-terminally pegylate the protein by performing the reaction at a pH which allows one to take advantage of the pK_a differences between the ε-amino group of the lysine residues and that of the α-amino group of the N-terminal residue of the protein. By such selective derivatization, attachment of a water soluble polymer to a protein is controlled: the conjugation with the polymer takes place predominantly at the N-terminus of the protein and no significant modification of other reactive groups, such as the lysine side chain amino groups, occurs. Using reductive alkylation, the water

soluble polymer may be of the type described above, and should have a single reactive aldehyde for coupling to the protein. Polyethylene glycol propionaldehyde, containing a single reactive aldehyde, may be used.

- 5 An N-terminally monopegylated derivative is preferred for ease in production of a therapeutic. N-terminal pegylation ensures a homogenous product as characterization of the product is simplified relative to di-, tri- or other multi pegylated products. The use
10 of the above reductive alkylation process for preparation of an N-terminal product is preferred for ease in commercial manufacturing.

Pharmaceutical Compositions

- 15 In yet another aspect of the present invention, methods of using pharmaceutical compositions of the proteins, and derivatives are provided. Such pharmaceutical compositions may be for administration by injection, or for oral, pulmonary, nasal, transdermal or
20 other forms of administration. In general, comprehended by the invention are pharmaceutical compositions comprising effective amounts of protein or derivative products of the invention together with pharmaceutically acceptable diluents, preservatives, solubilizers,
25 emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (e.g., Tris-HCl, acetate, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (e.g., Tween 80, Polysorbate 80), anti-oxidants
30 (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimersol, benzyl alcohol) and bulking substances (e.g., lactose, mannitol); incorporation of the material into particulate preparations of polymeric compounds such as polylactic
35 acid, polyglycolic acid, etc. or into liposomes.

Hyaluronic acid may also be used, and this may have the effect of promoting sustained duration in the circulation. Such compositions may influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the present proteins and derivatives. See, e.g., Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712 which are herein incorporated by reference. The compositions may be prepared in liquid form, or may be in dried powder, such as lyophilized form. Implantable sustained release formulations are also contemplated, as are transdermal formulations.

Contemplated for use herein are oral solid dosage forms, which are described generally in Remington's Pharmaceutical Sciences, 18th Ed. 1990 (Mack Publishing Co. Easton PA 18042) at Chapter 89, which is herein incorporated by reference. Solid dosage forms include tablets, capsules, pills, troches or lozenges, cachets or pellets. Also, liposomal or proteinoid encapsulation may be used to formulate the present compositions (e.g., proteinoid microspheres reported in U.S. Patent No. 4,925,673). Liposomal encapsulation may be used and the liposomes may be derivatized with various polymers (e.g. U.S. Patent No. 5,013,556). A description of possible solid dosage forms for the therapeutic is given by Marshall, K. In: *Modern Pharmaceutics* Edited by G.S. Banker and C.T. Rhodes Chapter 10, 1979, herein incorporated by reference. In general, the formulation will include the protein (or analog or derivative), and inert ingredients which allow for protection against the stomach environment, and release of the biologically active material in the intestine.

Also specifically contemplated are oral dosage forms of the above derivatized proteins. Protein may be

chemically modified so that oral delivery of the derivative is efficacious. Generally, the chemical modification contemplated is the attachment of at least one moiety to the protein (or peptide) molecule itself, where said moiety permits (a) inhibition of proteolysis; and (b) uptake into the blood stream from the stomach or intestine. Also desired is the increase in overall stability of the protein and increase in circulation time in the body. Examples of such moieties include: polyethylene glycol, copolymers of ethylene glycol and propylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone and polyproline. Abuchowski and Davis, Soluble polymer-Enzyme Adducts: "Enzymes as Drugs", Hoenberg and Roberts, eds., Wiley-Interscience, New York, NY, (1981), pp 367-383; Newmark, et al., J. Appl. Biochem. 4: 185-189 (1982). Other polymers that could be used are poly-1,3-dioxolane and poly-1,3,6-tioxocane.

For the protein (or derivative) the location of release may be the stomach, the small intestine (the duodenum, the jejunum, or the ileum), or the large intestine. One skilled in the art has available formulations which will not dissolve in the stomach, yet will release the material in the duodenum or elsewhere in the intestine. Preferably, the release will avoid the deleterious effects of the stomach environment, either by protection of the protein (or derivative) or by release of the biologically active material beyond the stomach environment, such as in the intestine.

To ensure full gastric resistance a coating impermeable to at least pH 5.0 is essential. Examples of the more common inert ingredients that are used as enteric coatings are cellulose acetate trimellitate (CAT), hydroxypropylmethylcellulose phthalate (HPMCP), HPMCP 50, HPMCP 55, polyvinyl acetate phthalate (PVAP),

Eudragit L30D, Aquateric, cellulose acetate phthalate (CAP), Eudragit L, Eudragit S, and Shellac. These coatings may be used as mixed films.

5 A coating or mixture of coatings can also be used on tablets, which are not intended for protection against the stomach. This can include sugar coatings, or coatings which make the tablet easier to swallow. Capsules may consist of a hard shell (such as gelatin) for delivery of dry therapeutic i.e. powder; for liquid
10 forms, a soft gelatin shell may be used. The shell material of cachets could be thick starch or other edible paper. For pills, lozenges, molded tablets or tablet triturates, moist massing techniques can be used.

The therapeutic can be included in the
15 formulation as fine multiparticulates in the form of granules or pellets of particle size about 1mm. The formulation of the material for capsule administration could also be as a powder, lightly compressed plugs or even as tablets. The therapeutic could be prepared by
20 compression.

Colorants and flavoring agents may all be included. For example, the protein (or derivative) may be formulated (such as by liposome or microsphere encapsulation) and then further contained within an
25 edible product, such as a refrigerated beverage containing colorants and flavoring agents.

One may dilute or increase the volume of the therapeutic with an inert material. These diluents could include carbohydrates, especially mannitol,
30 α -lactose, anhydrous lactose, cellulose, sucrose, modified dextrans and starch. Certain inorganic salts may be also be used as fillers including calcium triphosphate, magnesium carbonate and sodium chloride. Some commercially available diluents are Fast-Flo,
35 Emdex, STA-Rx 1500, Emcompress and Avicell.

Disintegrants may be included in the formulation of the therapeutic into a solid dosage form. Materials used as disintegrates include but are not limited to starch including the commercial disintegrant based on starch, Explotab. Sodium starch glycolate, Amberlite, sodium carboxymethylcellulose, ultramylopectin, sodium alginate, gelatin, orange peel, acid carboxymethyl cellulose, natural sponge and bentonite may all be used. Another form of the disintegrants are the insoluble cationic exchange resins. Powdered gums may be used as disintegrants and as binders and these can include powdered gums such as agar, Karaya or tragacanth. Alginic acid and its sodium salt are also useful as disintegrants.

Binders may be used to hold the therapeutic agent together to form a hard tablet and include materials from natural products such as acacia, tragacanth, starch and gelatin. Others include methyl cellulose (MC), ethyl cellulose (EC) and carboxymethyl cellulose (CMC). Polyvinyl pyrrolidone (PVP) and hydroxypropylmethyl cellulose (HPMC) could both be used in alcoholic solutions to granulate the therapeutic.

An antifrictional agent may be included in the formulation of the therapeutic to prevent sticking during the formulation process. Lubricants may be used as a layer between the therapeutic and the die wall, and these can include but are not limited to; stearic acid including its magnesium and calcium salts, polytetrafluoroethylene (PTFE), liquid paraffin, vegetable oils and waxes. Soluble lubricants may also be used such as sodium lauryl sulfate, magnesium lauryl sulfate, polyethylene glycol of various molecular weights, Carbowax 4000 and 6000.

Glidants that might improve the flow properties of the drug during formulation and to aid

rearrangement during compression might be added. The glidants may include starch, talc, pyrogenic silica and hydrated silicoaluminate.

To aid dissolution of the therapeutic into the aqueous environment a surfactant might be added as a wetting agent. Surfactants may include anionic detergents such as sodium lauryl sulfate, dioctyl sodium sulfosuccinate and dioctyl sodium sulfonate. Cationic detergents might be used and could include benzalkonium chloride or benzethonium chloride. The list of potential nonionic detergents that could be included in the formulation as surfactants are lauromacrogol 400, polyoxyl 40 stearate, polyoxyethylene hydrogenated castor oil 10, 50 and 60, glycerol monostearate, polysorbate 40, 60, 65 and 80, sucrose fatty acid ester, methyl cellulose and carboxymethyl cellulose. These surfactants could be present in the formulation of the protein or derivative either alone or as a mixture in different ratios.

Additives which potentially enhance uptake of the protein (or derivative) are for instance the fatty acids oleic acid, linoleic acid and linolenic acid.

Controlled release formulation may be desirable. The drug could be incorporated into an inert matrix which permits release by either diffusion or leaching mechanisms i.e. gums. Slowly degenerating matrices may also be incorporated into the formulation. Another form of a controlled release of this therapeutic is by a method based on the Oros therapeutic system (Alza Corp.), i.e. the drug is enclosed in a semipermeable membrane which allows water to enter and push drug out through a single small opening due to osmotic effects. Some entric coatings also have a delayed release effect.

Other coatings may be used for the formulation. These include a variety of sugars which could be applied in a coating pan. The therapeutic agent could also be given in a film coated tablet and the materials used in this instance are divided into 2 groups. The first are the nonenteric materials and include methyl cellulose, ethyl cellulose, hydroxyethyl cellulose, methylhydroxy-ethyl cellulose, hydroxypropyl cellulose, hydroxypropyl-methyl cellulose, sodium carboxy-methyl cellulose, providone and the polyethylene glycols. The second group consists of the enteric materials that are commonly esters of phthalic acid.

A mix of materials might be used to provide the optimum film coating. Film coating may be carried out in a pan coater or in a fluidized bed or by compression coating.

Also contemplated herein is pulmonary delivery of the present proteins, or derivative thereof. The protein (derivative) is delivered to the lungs of a mammal while inhaling and traverses across the lung epithelial lining to the blood stream. (Other reports of this include Adjei et al., Pharmaceutical Research 7: 565-569 (1990); Adjei et al., International Journal of Pharmaceutics 63: 135-144 (1990)(leuprolide acetate); Braquet et al., Journal of Cardiovascular Pharmacology 13(suppl. 5): s.143-146 (1989)(endothelin-1); Hubbard et al., Annals of Internal Medicine 3: 206-212 (1989)(α 1-antitrypsin); Smith et al., J. Clin. Invest. 84: 1145-1146 (1989)(α -1-proteinase); Oswein et al., "Aerosolization of Proteins", Proceedings of Symposium on Respiratory Drug Delivery II, Keystone, Colorado, March, 1990 (recombinant human growth hormone); Debs et al., The Journal of Immunology 140: 3482-3488 (1988)(interferon- γ and tumor necrosis factor alpha) and Platz et al., U.S.

Patent No. 5,284,656 (granulocyte colony stimulating factor).

Contemplated for use in the practice of this invention are a wide range of mechanical devices
5 designed for pulmonary delivery of therapeutic products, including but not limited to nebulizers, metered dose inhalers, and powder inhalers, all of which are familiar to those skilled in the art.

Some specific examples of commercially
10 available devices suitable for the practice of this invention are the Ultravent nebulizer, manufactured by Mallinckrodt, Inc., St. Louis, Missouri; the Acorn II nebulizer, manufactured by Marquest Medical Products, Englewood, Colorado; the Ventolin metered dose inhaler,
15 manufactured by Glaxo Inc., Research Triangle Park, North Carolina; and the Spinhaler powder inhaler, manufactured by Fisons Corp., Bedford, Massachusetts.

All such devices require the use of formulations suitable for the dispensing of protein (or
20 analog or derivative). Typically, each formulation is specific to the type of device employed and may involve the use of an appropriate propellant material, in addition to diluents, adjuvants and/or carriers useful in therapy.

25 The proteins (or derivative) should most advantageously be prepared in particulate form with an average particle size of less than 10 μm (or microns), most preferably 0.5 to 5 μm , for most effective delivery to the distal lung.

30 Carriers include carbohydrates such as trehalose, mannitol, xylitol, sucrose, lactose, and sorbitol. Other ingredients for use in formulations may include DPPC, DOPE, DSPC and DOPC. Natural or synthetic surfactants may be used. Polyethylene glycol
35 may be used (even apart from its use in derivatizing

the protein or analog). Dextrans, such as cyclodextran, may be used. Bile salts and other related enhancers may be used. Cellulose and cellulose derivatives may be used. Amino acids may be used, such as use in a buffer formulation.

Also, the use of liposomes, microcapsules or microspheres, inclusion complexes, or other types of carriers is contemplated.

Formulations suitable for use with a nebulizer, either jet or ultrasonic, will typically comprise protein (or derivative) dissolved in water at a concentration of about 0.1 to 25 mg of biologically active protein per mL of solution. The formulation may also include a buffer and a simple sugar (e.g., for protein stabilization and regulation of osmotic pressure). The nebulizer formulation may also contain a surfactant, to reduce or prevent surface induced aggregation of the protein caused by atomization of the solution in forming the aerosol.

Formulations for use with a metered-dose inhaler device will generally comprise a finely divided powder containing the protein (or derivative) suspended in a propellant with the aid of a surfactant. The propellant may be any conventional material employed for this purpose, such as a chlorofluorocarbon, a hydrochlorofluorocarbon, a hydrofluorocarbon, or a hydrocarbon, including trichlorofluoromethane, dichlorodifluoromethane, dichlorotetrafluoroethanol, and 1,1,1,2-tetrafluoroethane, or combinations thereof. Suitable surfactants include sorbitan trioleate and soya lecithin. Oleic acid may also be useful as a surfactant.

Formulations for dispensing from a powder inhaler device will comprise a finely divided dry powder

containing protein (or derivative) and may also include a bulking agent, such as lactose, sorbitol, sucrose, mannitol, trehalose, or xylitol in amounts which facilitate dispersal of the powder from the device, e.g.
5 50 to 90% by weight of the formulation.

Nasal delivery of the protein (or analog or derivative) is also contemplated. Nasal delivery allows the passage of the protein to the blood stream directly after administering the therapeutic product to the nose,
10 without the necessity for deposition of the product in the lung. Formulations for nasal delivery include those with dextran or cyclodextran. Delivery via transport across other mucus membranes is also contemplated.

One skilled in the art will be able to
15 ascertain effective dosages by administration and observing the desired therapeutic effect. Preferably, the formulation of the molecule or complex in a pharmaceutical composition will be such that between about .10 µg/kg/day and 10 µg/kg/day will yield the
20 desired therapeutic effect. The effective dosages may be determined using diagnostic tools over time. For example, a diagnostic for measuring the amount of OB or fibulin protein in the blood (or plasma or serum) may first be used to determine endogenous levels of OB or
25 fibulin protein. Such diagnostic tools may be in the form of an antibody assay, such as an antibody sandwich assay. The amount of OB protein can also be determined by means of the methods disclosed as part of the invention herein. These include incubating biological
30 samples with fibulin protein and determining the amount of OB protein bound in the OB/fibulin complex. The amount of endogenous OB or fibulin protein is quantified initially, and a baseline is determined. The therapeutic dosages are determined as the quantification
35 of endogenous and exogenous OB or fibulin protein (that

is, protein, analog or derivative found within the body, either self-produced or administered) is continued over the course of therapy. The dosages may therefore vary over the course of therapy, with a relatively high
5 dosage being used initially, until therapeutic benefit is seen, and lower dosages used to maintain the therapeutic benefits.

Methods of Use

10 Therapeutic benefits include the reduction of excess weight and/or excess fat. When excess weight and/or fat is reduced or eliminated, other therapeutic benefits may concomitantly occur, such as a treatment of Type II diabetes. There may also be a reduction in
15 blood lipid levels and related cardio-vascular problems, such as a reduction in LDL or VLDL, cholesterol, a reduction in arthral plaque or the rate of formation thereof, treatment of hyper-tension, and a reduction in the rate of gall stone formation.

20 Additional therapeutic benefits include the relative or absolute increase in lean mass along with loss in fat. Increase in lean tissue mass may also result in an increase in sensitivity to insulin, and improvement in overall strength, a decrease in bone
25 resorption, and an increase in red blood cells or oxygenation of tissue.

Even in the absence of weight loss, use of the present compositions may result in the above therapeutic benefits. There may be dosages administered for
30 maintenance of body weight or fat loss, but which result in the above therapeutic benefits.

Additionally, the present compositions may have solely cosmetic benefits in weight reduction or fat reduction.

The present methods may be used in conjunction with other medicaments, such as those useful for the treatment of diabetes (e.g., insulin, and possibly amylin), cholesterol and blood pressure lowering
5 medicaments (such as those which reduce blood lipid levels or other cardiovascular medicaments), and activity increasing medicaments (e.g., amphetamines). Appetite suppressants may also be used. Such administration may be simultaneous or may be in
10 seriatim.

In addition, the present methods may be used in conjunction with surgical procedures, such as cosmetic surgeries designed to alter the overall appearance of a body (e.g., liposuction or laser
15 surgeries designed to reduce body mass, or implant surgeries designed to increase the appearance of body mass). The health benefits of cardiac surgeries, such as bypass surgeries or other surgeries designed to relieve a deleterious condition caused by blockage of
20 blood vessels by fatty deposits, such as arterial plaque, may be increased with concomitant use of the present compositions and methods. Methods to eliminate gall stones, such as ultrasonic or laser methods, may also be used either prior to, during or after a course
25 of the present therapeutic methods. Furthermore, the present methods may be used as an adjunct to surgeries or therapies for broken bones, damaged muscle, or other therapies which would be improved by an increase in lean tissue mass.

30 Therefore, the present invention provides a method for treating disorders selected from among excess weight, high blood levels, high cholesterol, high triglycerides, Type II diabetes, or other related disorders, comprised of administering an effective

amount of fibulin type I protein selected from among the following:

- (a) the amino acid sequence 1 - 537 as set forth in SEQ. ID. NO. 7;
- 5 (b) the amino acid sequence 1 - 572 as set forth in SEQ. ID. NO. 8;
- (c) the amino acid sequence 1 - 654 as set forth in SEQ. ID. NO. 9;
- (d) the amino acid sequence 1 - 672 as set
10 forth in SEQ. ID. NO. 10;
- (e) the amino acid sequence of any of subparts (a), (b), (c), or (d) having a methionyl residue at the N-terminus;
- (f) the amino acid sequence of any of
15 subparts (a), (b), (c), or (d) having a different amino acid substituted in one or more of the following positions:
 - (i) one or more cysteine residues replaced by an alanine or serine residue;
 - 20 (ii) one or more tyrosine residues replaced by a phenylalanine residue;
 - (g) a truncated fibulin protein analog sufficient to bind an OB protein, analog or derivative thereof;
 - 25 (h) a hybrid molecule of fibulin protein selected from among the following:
 - (i) amino acids 1-537 and (connected to) amino acids 573-654 or amino acids 655-674;
 - (ii) amino acids 1-572 and (connected to)
30 amino acids 655-674;
 - (iii) amino acids 1-537 and (connected to) amino acids 573-654, having one or more of amino acids 538-572 placed between amino acids 537 and 573;

(iv) amino acids 1-537 and (connected to) amino acids 655-674, having one or more of amino acids 538-572 and/or 573-654, placed between amino acids 537 and 655; and

5 (v) amino acids 1-572 and (connected to) amino acids 655-674, having one or more of amino acids 573-654 placed between amino acids 572 and 655;

10 (i) the fibulin protein, analog or derivative of any of subparts (a) through (h) comprised of a chemical moiety connected to the protein moiety;

(j) a derivative of subpart (i) wherein said chemical moiety is a water soluble polymer moiety;

15 (k) a derivative of subpart (j) wherein said water soluble polymer moiety is polyethylene glycol;

(l) a derivative of subpart (j) wherein said water soluble polymer moiety is a polyamino acid moiety;

20 (m) a derivative of subpart (j) wherein said water soluble polymer moiety is attached at solely the N-terminus of said protein moiety;

(n) a combination of fibulin protein from any of subparts (a) through (m); and

(o) a dimer fibulin protein from any of subparts (a) through (m).

25

In addition, the present invention also provides a method for treating disorders selected from among excess weight, high blood levels, high cholesterol, high triglycerides, Type II diabetes, or
30 other related disorders comprised of administering a therapeutically effective amount of a complex consisting of fibulin type I, or analogs or derivatives thereof as discussed above, complexed to an OB protein, analog or derivative thereof selected from among the following:

- (a) the amino acid sequence 1-146 as set forth in SEQ. ID. NO. 3 (below) or SEQ ID. NO. 6 (below),
- (b) the amino acid sequence set 1-146 as
5 forth in SEQ. ID. NO. 6 (below) having a lysine residue at position 35 and an isoleucine residue at position 74;
- (c) the amino acid sequence of subpart (b) having a different amino acid substituted in one or more of the following positions (using the numbering
10 according to SEQ. ID. NO. 6, and retaining the same numbering even in the absence of a glutaminy residue at position 28): 4, 8, 32, 33, 35, 48, 50, 53, 60, 64, 66, 67, 68, 71, 74, 77, 78, 89, 97, 100, 102, 105, 106, 107, 108, 111, 112, 118, 136, 138, 142, and 145;
- 15 (d) the amino acid sequence of subparts (a), (b) or (c) optionally lacking a glutaminy residue at position 28;
- (e) the amino acid sequence of subparts (a), (b), (c), or (d) having a methionyl residue at the N
20 terminus.
- (f) a truncated OB protein analog selected from among: (using the numbering of SEQ. ID. NO. 6 having a lysine residue at position 35 and an isoleucine residue at position 74):
- 25 (i) amino acids 98-146;
(ii) amino acids 1-32;
(iii) amino acids 40-116;
(iv) amino acids 1-99 and 112-146;
(v) amino acids 1-99 and 112-146 having
30 one or more of amino acids 100-111 sequentially placed between amino acids 99 and 112;
(vi) the truncated OB analog of subpart (i) having one or more of amino acids 100, 102, 105, 106, 107, 108, 111, 112, 118, 136, 138, 142,
35 and 145 substituted with another amino acid;

(vii) the truncated analog of subpart (ii) having one or more of amino acids 4, 8 and 32 substituted with another amino acid;

5 (viii) the truncated analog of subpart (iii) having one or more of amino acids 50, 53, 60, 64, 66, 67, 68, 71, 74, 77, 78, 89, 97, 100, 102, 105, 106, 107, 108, 111 and 112 replaced with another amino acid;

10 (vix) the truncated analog of subpart (iv) having one or more of amino acids 4, 8, 32, 33, 35, 48, 50, 53, 60, 64, 66, 67, 68, 71, 74, 77, 78, 89, 97, 112, 118, 136, 138, 142, and 145 replaced with another amino acid;

15 (x) the truncated analog of subpart (v) having one or more of amino acids 4, 8, 32, 33, 35, 48, 50, 53, 60, 64, 66, 67, 68, 71, 74, 77, 78, 89, 97, 100, 102, 105, 106, 107, 108, 111, 112, 118, 136, 138, 142, and 145 replaced with another amino acid; and

20 (xi) the truncated analog of any of subparts (i)-(x) having an N-terminal methionyl residue;

(g) the OB protein or analog derivative of any of subparts (a) through (f) comprised of a chemical
25 moiety connected to the protein moiety;

(h) a derivative of subpart (g) wherein said chemical moiety is a water soluble polymer moiety;

(i) a derivative of subpart (h) wherein said water soluble polymer moiety is polyethylene glycol;

30 (j) A derivative of subpart (h) wherein said water soluble polymer moiety is a polyamino acid moiety;

(k) a derivative of subpart (h) wherein said water soluble polymer moiety is attached at solely the N-terminus of said protein moiety

(l) an OB protein, analog or derivative of any of subparts (a) through (k) in a pharmaceutically acceptable carrier.

Fibulin may also be used as an affinity
5 reagent to purify or detect the presence of OB protein in a biological sample. In one method, the fibulin protein is immobilized on CnBr-activated Sepharose and a column of protein-Sepharose conjugate is used to remove OB protein from liquid samples. In other methods,
10 Fibulin protein may also be immobilized on other support materials which are well known in the art. Fibulin can also be used as a diagnostic reagent to detect and quantitate OB protein in biological samples. Such methods, utilize fibulin protein as a capture agent
15 under conditions that allow OB protein in a biological sample to bind to fibulin protein forming a fibulin/OB complex. The amount of OB protein or the presence of OB protein in a sample can then be determined through known methods.

20

The following examples are offered to more fully illustrate the invention, but are not to be construed as limiting the scope thereof. Example 1 demonstrates that fibulin binds to OB protein. Example 2
25 is a prophetic example illustrating the use of the present fibulin pharmaceutical compositions for the treatment of obesity. Example 3 is a prophetic example illustrating the use of the present fibulin/OB protein complex pharmaceutical composition for the treatment of
30 obesity. Materials and Methods follow.

EXAMPLE 1

This Example demonstrates that fibulin binds to OB protein. Resin covalently bound to recombinant human OB
35 protein was contacted with human serum. The Resin/serum

mix was then washed to remove excess proteins. SDS-PAGE gel analysis and sequencing confirmation were then performed to identify fibulin/OB protein complex.

- 5 1. Resin preparation. Actigel-ALD (Sterogene), a Sepharose gel with activated aldehyde was used. Two small containers ("capsules") of resin were prepared. The first was a control resin, with the active groups (aldehyde) blocked by ethanolamine. The second was
10 resin covalently bound to recombinant human OB protein having the amino acid sequence set 1-146 as forth in SEQ. ID. NO. 6 (below) having a lysine residue at position 35 and an isoleucine residue at position 74, and a methionyl residue at position -1. Five milligrams
15 of OB protein was used per 200 microliters of resin.
2. Contact with human serum. The capsules of control or OB-resin were placed in human serum. For a typical study, 2000 milliliters of human serum was contacted
20 with 200 microliters of resin (either control or OB protein bound). The resin/serum mix was allowed to stir for 48 hours at 4°C.
3. First Wash. The resin capsule was removed from the
25 serum, and washed until the eluant was relatively protein-free, having an OD below .01 at A₂₈₀. The first was buffer contained 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM CaCl₂ and 2 mM MgCl₂. One milliliter fractions were collected.
30
4. EDTA Elution. The resin capsule was then washed with the same buffer, but containing no MgCl₂ or CaCl₂, and containing 10 mM EDTA. One milliliter fractions were collected and O.D. nm monitored to follow protein
35 elution.

5. GEB Elution. Gentle Antibody/Antigen Elution Buffer ("GEB", Pierce Chemical) was used to wash the resin capsule, and one milliliter fractions were collected.

5 The fractions were combined, and dialyzed against the above Tris buffer containing metals, or against phosphate buffered saline.

6. SDS-PAGE. The SDS-PAGE (non-reducing conditions) results are shown in FIGURE 1. Lane 4 contains molecular weight standards ranging from 200 kD to 6kD. The presence of fibulin binding to OB protein is demonstrated by comparing lane 1 containing the EDTA elution from the control resin (first lane on the left) and the lane 2, containing the EDTA elution from the OB-linked resin (second lane from the left). As can be seen, there is a band at approximately 85 kD seen in lane 2. Fibulin dimer binding to OB protein is demonstrated by comparing lane 1 containing the EDTA elutions from the control resin and lane 2, containing the EDTA elution from the OB-linked resin. As can be seen, there is a band at approxiamtely 200 kD in lane 2.

7. Sequencing Confirmation. Sequencing confirmed the identity of fibulin in the 85 kD band. The N-terminal 13 amino acids were identical to the N-terminal amino acids of the mature fibulin protein (GenBank accession No. P37888).

30 EXAMPLE 2

This Example demonstrates the use of fibulin pharmaceutical compositions for the treatment of obesity.

35 An obese human, with a BMI greater than 30, is treated with an effective dose of fibulin in a

pharmaceutically acceptable diluent, adjuvant or carrier. The fibulin is selected from amino acids (variant A) 1-537 of SEQ. ID. NO. 7; amino acids (variant B) 1-572 of SEQ. ID. NO. 8; amino acids
5 (variant C) 1-654 of SEQ. ID. NO. 9; amino acids (variant D) 1-674 of SEQ. ID. NO. 10 or other analogs or derivatives discussed herein. During the course of treatment, fibulin levels in the serum are monitored using an anti-fibulin selective binding molecule, such
10 as a monoclonal antibody.

EXAMPLE 3

This Example demonstrates the use of fibulin/OB protein complex pharmaceutical compositions
15 for the treatment of obesity.

An obese human, with a BMI greater than 30, is treated with an effective dose of fibulin/OB protein complex, in a pharmaceutically acceptable diluent, adjuvant or carrier. The fibulin and OB protein are
20 selected from the amino acids, analogs and derivatives described herein. The fibulin can be monitored using an anti-fibulin selective binding molecule, such as a monoclonal antibody.

25 METHODS FOR PRODUCTION OF FIBULIN TYPE 1

Biologically active recombinant fibulin type 1 can be produced using standard methods well known in the art. In addition, fibulin type 1 can also be isolated and
30 purified from naturally occurring sources such as human placenta or human serum or plasma using standard methods well known in the art. Such methods have previously been described in Balbona et al., J. Biol. Chem. 267: 20120-20125 (1992) and Argaves et al., J. Cell Biol. 111: 3155-
35 3164 (1990). As discussed previously herein, standard

methods for producing methionyl analogs and other derivatives can also be used in the preparation of fibulin type 1 analogs or derivatives.

5 METHODS FOR PRODUCTION OF OB PROTEIN

The below methods for production have been used to produce biologically active recombinant methionyl murine or human analog OB protein. Similar methods may be used to prepare biologically active recombinant methionyl human OB
10 protein.

Expression Vector and Host Strain

The plasmid expression vector used is pCFM1656, ATCC Accession No. 69576. The above DNA was ligated into the expression vector pCFM1656 linearized
15 with XbaI and BamHI and transformed into the *E. coli* host strain, FM5. *E. coli* FM5 cells were derived at Amgen Inc., Thousand Oaks, CA from *E. coli* K-12 strain (Bachmann, et al., Bacteriol. Rev. 40: 116-167 (1976)) and contain the integrated lambda phage repressor gene,
20 cI857 (Sussman et al., C.R. Acad. Sci. 254: 1517-1579 (1962)). Vector production, cell transformation, and colony selection were performed by standard methods. E.g., Sambrook, et al., Molecular Cloning: A Laboratory Manual, 2d Edition, Cold Spring Harbor Laboratory Press,
25 Cold Spring Harbor, NY. Host cells were grown in LB media.

Fermentation Process A three-phase fermentation protocol known as a fed-batch process was used. Media compositions are set forth below.

30 Batch: A nitrogen and phosphate source were sterilized (by raising to 122 °C for 35 minutes, 18-20 psi) in the fermentation vessel (Biolafitte, 12 liter capacity). Upon cooling, carbon, magnesium, vitamin, and trace metal sources were added aseptically. An
35 overnight culture of the above recombinant murine

protein-producing bacteria (16 hours or more) of 500 mL (grown in LB broth) was added to the fermentor.

Feed I: Upon reaching between 4.0-6.0 OD₆₀₀, cultures were fed with Feed I. The glucose was fed at a limiting rate in order to control the growth rate (μ). An automated system (called the Distributive Control System) was instructed to control the growth rate to 0.15 generations per hour.

Feed II: When the OD₆₀₀ had reached 30, culture temperature were slowly increased to 42°C and the feed changed to Feed II, below. The fermentation was allowed to continue for 10 hours with sampling every 2 hours. After 10 hours, the contents of the fermentor was chilled to below 20°C and harvested by centrifugation.

Media Composition:

20	Batch:	10 g/L	Yeast extract
		5.25 g/L	(NH ₄) ₂ SO ₄
		3.5 g/L	K ₂ HPO ₄
		4.0 g/L	KH ₂ PO ₄
		5.0 g/L	Glucose
		1.0 g/L	MgSO ₄ ·7H ₂ O
25		2.0 mL/L	Vitamin Solution
		2.0 mL/L	Trace Metal Solution
		1.0 mL/L	P2000 Antifoam
30	Feed I:	50 g/L	Bacto-tryptone
		50 g/L	Yeast extract
		450 g/L	Glucose
		8.75 g/L	MgSO ₄ ·7H ₂ O
		10 mL/L	Vitamin Solution
		10 mL/L	Trace Metal Solution
35	Feed II:	200 g/L	Bacto-tryptone
		100 g/L	Yeast extract
		110 g/L	Glucose

Vitamin Solution (Batch and Feed I):

0.5 g Biotin, 0.4 g Folic acid, and 4.2 g riboflavin,
was dissolved in 450 mls H₂O and 3 mls 10 N NaOH, and
brought to 500 mLs in H₂O. 14 g pyridoxine-HCl and 61 g
5 niacin was dissolved 150 ml H₂O and 50 ml 10 N NaOH, and
brought to 250 ml in H₂O. 54 g pantothenic acid was
dissolved in 200 mL H₂O, and brought to 250 mL. The
three solutions were combined and brought to 10 liters
total volume.

10

Trace Metal Solution (Batch and Feed I):

Ferric Chloride (FeCl₃·6H₂O): 27 g/L
Zinc Chloride (ZnCl₂·4H₂O): 2 g/L
Cobalt Chloride (CoCl₂·6H₂O): 2 g/L
15 Sodium Molybdate (NaMoO₄·2H₂O): 2 g/L
Calcium Chloride (CaCl₂·2H₂O): 1 g/L
Cupric Sulfate (CuSO₄·5H₂O): 1.9 g/L
Boric Acid (H₃BO₃): 0.5 g/L
Manganese Chloride (MnCl₂·4H₂O): 1.6 g/L
20 Sodium Citrate dihydrate: 73.5 g/L

Purification Process for Murine OB Protein

Purification was accomplished by the following
steps (unless otherwise noted, the following steps were
25 performed at 4°C):

1. Cell paste. *E. coli* cell paste was suspended
in 5 times volume of 7 mM of EDTA, pH 7.0. The cells in
the EDTA were further broken by two passes through a
microfluidizer. The broken cells were centrifuged at
30 4.2 K rpm for 1 hour in a Beckman J6-B centrifuge with a
JS-4.2 rotor.

2. Inclusion body wash #1. The supernatant from
above was removed, and the pellet was resuspended with
5 times volume of 7 mM EDTA, pH 7.0, and homogenized.
35 This mixture was centrifuged as in step 1.

3. Inclusion body wash #2. The supernatant from above was removed, and the pellet was resuspended in ten times volume of 20 mM tris, pH 8.5, 10 mM DTT, and 1% deoxycholate, and homogenized. This mixture was
5 centrifuged as in step 1.

4. Inclusion body wash #3. The supernatant from above was removed and the pellet was resuspended in ten times volume of distilled water, and homogenized. This mixture was centrifuged as in step 1.

10 5. Refolding. The pellet was refolded with 15 volumes of 10 mM HEPES, pH 8.5, 1% sodium sarcosine (N-lauroyl sarcosine), at room temperature. After 60 minutes, the solution was made to be 60 μ M copper sulfate, and then stirred overnight.

15 6. Removal of sarcosine. The refolding mixture was diluted with 5 volumes of 10 mM tris buffer, pH 7.5, and centrifuged as in step 1. The supernatant was collected, and mixed with agitation for one hour with Dowex® 1-X4 resin (Dow Chemical Co., Midland MI), 20-50
20 mesh, chloride form, at 0.066% total volume of diluted refolding mix. See WO 89/10932 at page 26 for more information on Dowex®. This mixture was poured into a column and the eluant collected. Removal of sarcosine was ascertained by reverse phase HPLC.

25 7. Acid precipitation. The eluant from the previous step was collected, and pH adjusted to pH 5.5, and incubated for 30 minutes at room temperature. This mixture was centrifuged as in step 1.

8. Cation exchange chromatography. The pH of the
30 supernatant from the previous step was adjusted to pH 4.2, and loaded on CM Sepharose Fast Flow (at 7% volume). 20 column volumes of salt gradient were done at 20 mM NaOAC, pH 4.2, 0 M to 1.0 M NaCl.

9. Hydrophobic interaction chromatography. The CM
35 Sepharose pool of peak fractions (ascertained from

ultraviolet absorbance) from the above step was made to be 0.2 M ammonium sulfate. A 20 column volume reverse salt gradient was done at 5 mM NaOAC, pH 4.2, with .4 M to 0 M ammonium sulfate. This material was concentrated and diafiltered into PBS.

Fermentation of recombinant human OB protein analog.

Fermentation of the above host cells to produce recombinant human OB protein analog (SEQ. ID. NO. 4) can be accomplished using the conditions and compositions as described above for recombinant murine material.

Purification of the recombinant human OB protein analog.

Recombinant human protein analog may be purified using methods similar to those used for purification of recombinant murine protein. For preparation of recombinant human OB protein analog, step 8 should be performed by adjusting the pH of the supernatant from step 7 to pH 5.0, and loading this onto a CM Sepharose fast flow column. The 20 column volume salt gradient should be performed at 20 mM NaOAC, pH 5.5, 0M to 0.5 M NaCl. Step 9 should be performed by diluting the CM Sepharose pool four fold with water, and adjusting the pH to 7.5. This mixture should be made to 0.7 M ammonium sulfate. Twenty column volume reverse salt gradient should be done at 5 mM NaOAC, pH 5.5, 0.2 M to 0M ammonium sulfate. Otherwise, the above steps are identical. Recombinant human OB protein of SEQ.ID.NO.6 having lysine 35 and isoleucine 74 can be formulated in a buffer containing 10 mM histidine, 4.3% arginine, at pH 6.0.

Methods of producing fibulin Type 1/OB Protein Complex.

Biological active recombinant fibulin type 1 or recombinant OB protein can be produced according to the above methods. Fibulin type 1/OB protein complex can be prepared by admixing purified and isolated Fibulin type 1 with purified and isolated OB protein. The fibulin type 1/OB protein complex will form due to the ability of fibulin type 1 to associate with OB protein.

While the present invention has been described in terms of preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations which come within the scope of the invention as claimed.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: AMGEN INC.
- (ii) TITLE OF INVENTION: FIBULIN PHARMACEUTICAL COMPOSITIONS AND RELATED METHODS
- (iii) NUMBER OF SEQUENCES: 10
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: AMGEN INC.
 - (B) STREET: 1840 DEHAVILLAND DRIVE
 - (C) CITY: THOUSAND OAKS
 - (D) STATE: CA
 - (E) COUNTRY: USA
 - (F) ZIP: 91320-1789
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/627,636
 - (B) FILING DATE: 04-APR-1996
 - (C) CLASSIFICATION: NOT ASSIGNED YET
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Knight, Matthew W.
 - (B) REGISTRATION NUMBER: 36,846
 - (C) REFERENCE/DOCKET NUMBER: A-389

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 491 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TCTAGATTTG AGTTTAACT TTTAGAAGGA GGAATAACAT ATGGTACCGA TCCAGAAAGT	60
TCAGGACGAC ACCAAAACCT TAATTAAAC GATCGTTACG CGTATCAACG ACATCAGTCA	120
CACCCAGTCG GTCTCCGCTA AACAGCGTGT TACCGGTCTG GACTTCATCC CGGGTCTGCA	180

-44-

CCCGATCCTA AGCTTGTCCTA AAATGGACCA GACCCTGGCT GTATACCAGC AGGTGTTAAC	240
CTCCCTGCCG TCCCAGAACG TTCTTCAGAT CGCTAACGAC CTCGAGAACC TTCGCGACCT	300
GCTGCACCTG CTGGCATTCT CCAAATCCTG CTCCCTGCCG CAGACCTCAG GTCTTCAGAA	360
ACCGGAATCC CTGGACGGGG TCCTGGAAGC ATCCCTGTAC AGCACCGAAG TTGTTGCTCT	420
GTCCCGTCTG CAGGGTTCCC TTCAGGACAT CCTTCAGCAG CTGGACGTTT CTCCGGAATG	480
TTAATGGATC C	491

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 491 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGATCTAAAC TCAAAATTGA AAATCTTCCT CCTTATTGTA TACCATGGCT AGGTCTTTCA	60
AGTCCTGCTG TGGTTTTGGA ATTAATTTTG CTAGCAATGC GCATAGTTGC TG TAGTCAGT	120
GTGGGTCAGC CAGAGGCGAT TTGTCGCACA ATGGCCAGAC CTGAAGTAGG GCCCAGACGT	180
GGGCTAGGAT TCGAACAGGT TTTACCTGGT CTGGGACCGA CATATGGTCG TCCACAATTG	240
GAGGGACGGC AGGGTCTTGC AAGAAGTCTA GCGATTGCTG GAGCTCTTGG AAGCGCTGGA	300
CGACGTGGAC GACCGTAAGA GGTTTAGGAC GAGGGACGGC GTCTGGAGTC CAGAAGTCTT	360
TGGCCTTAGG GACCTGCCCC AGGACCTTCG TAGGGACATG TCGTGGCTTC AACAAACGAGA	420
CAGGGCAGAC GTCCCAAGGG AAGTCCTGTA GGAAGTCGTC GACCTGCAAA GAGGCCTTAC	480
AATTACCTAG G	491

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 147 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

-45-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```

Met Val Pro Ile Gln Lys Val Gln Asp Asp Thr Lys Thr Leu Ile Lys
1           5           10           15
Thr Ile Val Thr Arg Ile Asn Asp Ile Ser His Thr Gln Ser Val Ser
           20           25           30
Ala Lys Gln Arg Val Thr Gly Leu Asp Phe Ile Pro Gly Leu His Pro
           35           40           45
Ile Leu Ser Leu Ser Lys Met Asp Gln Thr Leu Ala Val Tyr Gln Gln
           50           55           60
Val Leu Thr Ser Leu Pro Ser Gln Asn Val Leu Gln Ile Ala Asn Asp
65           70           75           80
Leu Glu Asn Leu Arg Asp Leu Leu His Leu Leu Ala Phe Ser Lys Ser
           85           90           95
Cys Ser Leu Pro Gln Thr Ser Gly Leu Gln Lys Pro Glu Ser Leu Asp
           100          105          110
Gly Val Leu Glu Ala Ser Leu Tyr Ser Thr Glu Val Val Ala Leu Ser
           115          120          125
Arg Leu Gln Gly Ser Leu Gln Asp Ile Leu Gln Gln Leu Asp Val Ser
           130          135          140
Pro Glu Cys
145

```

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 454 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

CATATGGTAC CGATCCAGAA AGTTCAGGAC GACACCAAAA CCTTAATTAA AACGATCGTT      60
ACGCGTATCA ACGACATCAG TCACACCCAG TCGGTGAGCT CTAAACAGCG TGTACAGGC      120
CTGGACTTCA TCCCGGGTCT GCACCCGATC CTGACCTTGT CCAAATGGA CCAGACCCTG      180
GCTGTATACC AGCAGATCTT AACCTCCATG CCGTCCCGTA ACGTTCTTCA GATCTCTAAC      240
GACCTCGAGA ACCTTCGCGA CCTGCTGCAC GTGCTGGCAT TCTCCAAATC CTGCCACCTG      300
CCATGGGCTT CAGGTCTTGA GACTCTGGAC TCTCTGGGCG GGGTCCTGGA AGCATCCGGT      360

```

-46-

TACAGCACCG AAGTTGTTGC TCTGTCCCGT CTGCAGGGTT CCCTTCAGGA CATGCTTTGG 420

CAGCTGGACC TGTCTCCGGG TTGTTAATGG ATCC 454

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 454 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTATACCATG GCTAGGTCTT TCAAGTCCTG CTGTGGTTTT GGAATTAATT TTGCTAGCAA 60

TGCGCATAGT TGCTGTAGTC AGTGTGGGTC AGCCACTCGA GATTTGTCGC ACAATGTCCG 120

GACCTGAAGT AGGGCCCAGA CGTGGGCTAG GACTGGAACA GGTTTTACCT GGTCTGGGAC 180

CGACATATGG TCGTCTAGAA TTGGAGGTAC GGCAGGGCAT TGCAAGAAGT CTAGAGATTG 240

CTGGAGCTCT TGGAAGCGCT GGACGACGTG CACGACCGTA AGAGGTTTAG GACGGTGGAC 300

GGTACCCGAA GTCCAGAACT CTGAGACCTG AGAGACCCGC CCCAGGACCT TCGTAGGCCA 360

ATGTCGTGGC TTCAACAACG AGACAGGGCA GACGTCCCAA GGAAGTCCT GTACGAAACC 420

GTCGACCTGG ACAGAGGCCC AACAATTACC TAGG 454

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 147 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Val Pro Ile Gln Lys Val Gln Asp Asp Thr Lys Thr Leu Ile Lys
 1 5 10 15

Thr Ile Val Thr Arg Ile Asn Asp Ile Ser His Thr Gln Ser Val Ser
 20 25 30

-47-

Ser Lys Gln Arg Val Thr Gly Leu Asp Phe Ile Pro Gly Leu His Pro
 35 40 45
 Ile Leu Thr Leu Ser Lys Met Asp Gln Thr Leu Ala Val Tyr Gln Gln
 50 55 60
 Ile Leu Thr Ser Met Pro Ser Arg Asn Val Leu Gln Ile Ser Asn Asp
 65 70 75 80
 Leu Glu Asn Leu Arg Asp Leu Leu His Val Leu Ala Phe Ser Lys Ser
 85 90 95
 Cys His Leu Pro Trp Ala Ser Gly Leu Glu Thr Leu Asp Ser Leu Gly
 100 105 110
 Gly Val Leu Glu Ala Ser Gly Tyr Ser Thr Glu Val Val Ala Leu Ser
 115 120 125
 Arg Leu Gln Gly Ser Leu Gln Asp Met Leu Trp Gln Leu Asp Leu Ser
 130 135 140
 Pro Gly Cys
 145

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 556 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

- (ix) FEATURE: LEADER SEQUENCE=POSITIONS 1-29. SEE FIGURE 7 AND CORRESPONDING DESCRIPTION.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met Glu Arg Ala Ala Pro Ser Arg Arg Val Pro Leu Pro Leu Leu Leu
 1 5 10 15
 Leu Gly Gly Leu Ala Leu Leu Ala Ala Gly Val Asp Ala Asp Val Leu
 20 25 30
 Leu Glu Ala Cys Cys Ala Asp Gly His Arg Met Ala Thr His Gln Lys
 35 40 45
 Asp Cys Ser Leu Pro Tyr Ala Thr Glu Ser Lys Glu Cys Arg Met Val
 50 55 60
 Gln Glu Gln Cys Cys His Ser Gln Leu Glu Glu Leu His Cys Ala Thr
 65 70 75 80
 Cys Ala Thr Pro His Gly Asp Asn Ala Ser Leu Glu Ala Thr Phe Val
 85 90 95

-48-

Lys Arg Cys Cys His Cys Cys Leu Leu Gly Arg Ala Ala Gln Ala Gln
 100 105 110
 Gly Gln Ser Cys Glu Tyr Ser Leu Met Val Gly Tyr Gln Cys Gly Gln
 115 120 125
 Val Phe Arg Ala Cys Cys Val Lys Ser Gln Glu Thr Gly Asp Leu Asp
 130 135 140
 Val Gly Gly Leu Gln Glu Thr Asp Lys Ile Ile Glu Val Glu Glu Glu
 145 150 155 160
 Gln Glu Asp Pro Tyr Leu Asn Asp Arg Cys Arg Gly Gly Gly Pro Cys
 165 170 175
 Lys Gln Gln Cys Arg Asp Thr Gly Asp Glu Val Val Cys Ser Cys Phe
 180 185 190
 Val Gly Tyr Gln Leu Leu Ser Asp Gly Val Ser Cys Glu Asp Val Asn
 195 200 205
 Glu Cys Ile Thr Gly Ser His Ser Cys Arg Leu Gly Glu Ser Cys Ile
 210 215 220
 Asn Thr Val Gly Ser Phe Arg Cys Gln Arg Asp Ser Ser Cys Gly Thr
 225 230 235 240
 Gly Tyr Glu Leu Thr Glu Asp Asn Ser Cys Lys Asp Ile Asp Glu Cys
 245 250 255
 Glu Ser Gly Ile His Asn Cys Leu Pro Asp Phe Ile Cys Gln Asn Thr
 260 265 270
 Leu Gly Ser Phe Arg Cys Arg Pro Lys Leu Gln Cys Lys Ser Gly Phe
 275 280 285
 Ile Gln Asp Ala Leu Gly Asn Cys Ile Asp Ile Asn Glu Cys Leu Ser
 290 295 300
 Ile Ser Ala Pro Cys Pro Ile Gly His Thr Cys Ile Asn Thr Glu Gly
 305 310 315 320
 Ser Tyr Thr Cys Gln Lys Asn Val Pro Asn Cys Gly Arg Gly Tyr His
 325 330 335
 Leu Asn Glu Glu Gly Thr Arg Cys Val Asp Val Asp Glu Cys Ala Pro
 340 345 350
 Pro Ala Glu Pro Cys Gly Lys Gly His Arg Cys Val Asn Ser Pro Gly
 355 360 365
 Ser Phe Arg Cys Glu Cys Lys Thr Gly Tyr Tyr Phe Asp Gly Ile Ser
 370 375 380
 Arg Met Cys Val Asp Val Asn Glu Cys Gln Arg Tyr Pro Gly Arg Leu
 385 390 395 400
 Cys Gly His Lys Cys Glu Asn Thr Leu Gly Ser Tyr Leu Cys Ser Cys

405	410	415
Ser Val Gly Phe Arg Leu Ser Val Asp Gly Arg Ser Cys Glu Asp Ile		
420	425	430
Asn Glu Cys Ser Ser Ser Pro Cys Ser Gln Glu Cys Ala Asn Val Tyr		
435	440	445
Gly Ser Tyr Gln Cys Tyr Cys Arg Arg Gly Tyr Gln Leu Ser Asp Val		
450	455	460
Asp Gly Val Thr Cys Glu Asp Ile Asp Glu Cys Ala Leu Pro Thr Gly		
465	470	475
Gly His Ile Cys Ser Tyr Arg Cys Ile Asn Ile Pro Gly Ser Phe Gln		
485	490	495
Cys Ser Cys Pro Ser Ser Gly Tyr Arg Leu Ala Pro Asn Gly Arg Asn		
500	505	510
Cys Gln Asp Ile Asp Glu Cys Val Thr Gly Ile His Asn Cys Ser Ile		
515	520	525
Asn Glu Thr Cys Phe Asn Ile Gln Gly Ala Phe Arg Cys Leu Ala Phe		
530	535	540
Glu Cys Pro Glu Asn Tyr Arg Arg Ser Ala Ala Thr		
545	550	555

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 601 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

- (ix) FEATURE: LEADER SEQUENCE=POSITIONS 1-29. SEE FIGURE 8 AND CORRESPONDING DESCRIPTION.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Glu Arg Ala Ala Pro Ser Arg Arg Val Pro Leu Pro Leu Leu Leu		
1	5	10
Leu Gly Gly Leu Ala Leu Leu Ala Ala Gly Val Asp Ala Asp Val Leu		
20	25	30
Leu Glu Ala Cys Cys Ala Asp Gly His Arg Met Ala Thr His Gln Lys		
35	40	45
Asp Cys Ser Leu Pro Tyr Ala Thr Glu Ser Lys Glu Cys Arg Met Val		
50	55	60
Gln Glu Gln Cys Cys His Ser Gln Leu Glu Glu Leu His Cys Ala Thr		
65	70	75
		80

-51-

Lys Thr Gly Tyr Tyr Phe Asp Gly Ile Ser Arg Met Cys Val Asp Val
 385 390 395 400
 Asn Glu Cys Gln Arg Tyr Pro Gly Arg Leu Cys Gly His Lys Cys Glu
 405 410 415
 Asn Thr Leu Gly Ser Tyr Leu Cys Ser Cys Ser Val Gly Phe Arg Leu
 420 425 430
 Ser Val Asp Gly Arg Ser Cys Glu Asp Ile Asn Glu Cys Ser Ser Ser
 435 440 445
 Pro Cys Ser Gln Glu Cys Ala Asn Val Tyr Gly Ser Tyr Gln Cys Tyr
 450 455 460
 Cys Arg Arg Gly Tyr Gln Leu Ser Asp Val Asp Gly Val Thr Cys Glu
 465 470 475 480
 Asp Ile Asp Glu Cys Ala Leu Pro Thr Gly Gly His Ile Cys Ser Tyr
 485 490 495
 Arg Cys Ile Asn Ile Pro Gly Ser Phe Gln Cys Ser Cys Pro Ser Ser
 500 505 510
 Gly Tyr Arg Leu Ala Pro Asn Gly Arg Asn Cys Gln Asp Ile Asp Glu
 515 520 525
 Cys Val Thr Gly Ile His Asn Cys Ser Ile Asn Glu Thr Cys Phe Asn
 530 535 540
 Ile Gln Gly Ala Phe Arg Cys Leu Ala Phe Glu Cys Pro Glu Asn Tyr
 545 550 555 560
 Arg Arg Ser Ala Ala Thr Gln Lys Ser Lys Lys Gly Arg Gln Asn Thr
 565 570 575
 Pro Ala Gly Ser Ser Lys Glu Asp Cys Arg Val Leu Pro Trp Lys Gln
 580 585 590
 Gly Leu Glu Asp Thr His Leu Asp Ala
 595 600

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 683 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

- (ix) FEATURE: LEADER SEQUENCE=POSITIONS 1-29. SEE FIGURE 9 AND CORRESPONDING DESCRIPTION.

-52-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Met	Glu	Arg	Ala	Ala	Pro	Ser	Arg	Arg	Val	Pro	Leu	Pro	Leu	Leu	Leu	1	5	10	15
Leu	Gly	Gly	Leu	Ala	Leu	Leu	Ala	Ala	Gly	Val	Asp	Ala	Asp	Val	Leu	20	25	30	
Leu	Glu	Ala	Cys	Cys	Ala	Asp	Gly	His	Arg	Met	Ala	Thr	His	Gln	Lys	35	40	45	
Asp	Cys	Ser	Leu	Pro	Tyr	Ala	Thr	Glu	Ser	Lys	Glu	Cys	Arg	Met	Val	50	55	60	
Gln	Glu	Gln	Cys	Cys	His	Ser	Gln	Leu	Glu	Glu	Leu	His	Cys	Ala	Thr	65	70	75	80
Gly	Ile	Ser	Leu	Ala	Asn	Glu	Gln	Asp	Arg	Cys	Ala	Thr	Pro	His	Gly	85	90	95	
Asp	Asn	Ala	Ser	Leu	Glu	Ala	Thr	Phe	Val	Lys	Arg	Cys	Cys	His	Cys	100	105	110	
Cys	Leu	Leu	Gly	Arg	Ala	Ala	Gln	Ala	Gln	Gly	Gln	Ser	Cys	Glu	Tyr	115	120	125	
Ser	Leu	Met	Val	Gly	Tyr	Gln	Cys	Gly	Gln	Val	Phe	Arg	Ala	Cys	Cys	130	135	140	
Val	Lys	Ser	Gln	Glu	Thr	Gly	Asp	Leu	Asp	Val	Gly	Gly	Leu	Gln	Glu	145	150	155	160
Thr	Asp	Lys	Ile	Ile	Glu	Val	Glu	Glu	Glu	Gln	Glu	Asp	Pro	Tyr	Leu	165	170	175	
Asn	Asp	Arg	Cys	Arg	Gly	Gly	Gly	Pro	Cys	Lys	Gln	Gln	Cys	Arg	Asp	180	185	190	
Thr	Gly	Asp	Glu	Val	Val	Cys	Ser	Cys	Phe	Val	Gly	Tyr	Gln	Leu	Leu	195	200	205	
Ser	Asp	Gly	Val	Ser	Cys	Glu	Asp	Val	Asn	Glu	Cys	Ile	Thr	Gly	Ser	210	215	220	
His	Ser	Cys	Arg	Leu	Gly	Glu	Ser	Cys	Ile	Asn	Thr	Val	Gly	Ser	Phe	225	230	235	240
Arg	Cys	Gln	Arg	Asp	Ser	Ser	Cys	Gly	Thr	Gly	Tyr	Glu	Leu	Thr	Glu	245	250	255	
Asp	Asn	Ser	Cys	Lys	Asp	Ile	Asp	Glu	Cys	Glu	Ser	Gly	Ile	His	Asn	260	265	270	
Cys	Leu	Pro	Asp	Phe	Ile	Cys	Gln	Asn	Thr	Leu	Gly	Ser	Phe	Arg	Cys	275	280	285	
Arg	Pro	Lys	Leu	Gln	Cys	Lys	Ser	Gly	Phe	Ile	Gln	Asp	Ala	Leu	Gly	290	295	300	

-53-

Asn Cys Ile Asp Ile Asn Glu Cys Leu Ser Ile Ser Ala Pro Cys Pro
 305 310 315 320
 Ile Gly His Thr Cys Ile Asn Thr Glu Gly Ser Tyr Thr Cys Gln Lys
 325 330 335
 Asn Val Pro Asn Cys Gly Arg Gly Tyr His Leu Asn Glu Glu Gly Thr
 340 345 350
 Arg Cys Val Asp Val Asp Glu Cys Ala Pro Pro Ala Glu Pro Cys Gly
 355 360 365
 Lys Gly His Arg Cys Val Asn Ser Pro Gly Ser Phe Arg Cys Glu Cys
 370 375 380
 Lys Thr Gly Tyr Tyr Phe Asp Gly Ile Ser Arg Met Cys Val Asp Val
 385 390 395 400
 Asn Glu Cys Gln Arg Tyr Pro Gly Arg Leu Cys Gly His Lys Cys Glu
 405 410 415
 Asn Thr Leu Gly Ser Tyr Leu Cys Ser Cys Ser Val Gly Phe Arg Leu
 420 425 430
 Ser Val Asp Gly Arg Ser Cys Glu Asp Ile Asn Glu Cys Ser Ser Ser
 435 440 445
 Pro Cys Ser Gln Glu Cys Ala Asn Val Tyr Gly Ser Tyr Gln Cys Tyr
 450 455 460
 Cys Arg Arg Gly Tyr Gln Leu Ser Asp Val Asp Gly Val Thr Cys Glu
 465 470 475 480
 Asp Ile Asp Glu Cys Ala Leu Pro Thr Gly Gly His Ile Cys Ser Tyr
 485 490 495
 Arg Cys Ile Asn Ile Pro Gly Ser Phe Gln Cys Ser Cys Pro Ser Ser
 500 505 510
 Gly Tyr Arg Leu Ala Pro Asn Gly Arg Asn Cys Gln Asp Ile Asp Glu
 515 520 525
 Cys Val Thr Gly Ile His Asn Cys Ser Ile Asn Glu Thr Cys Phe Asn
 530 535 540
 Ile Gln Gly Ala Phe Arg Cys Leu Ala Phe Glu Cys Pro Glu Asn Tyr
 545 550 555 560
 Arg Arg Ser Ala Ala Thr Arg Cys Glu Arg Leu Pro Cys His Glu Asn
 565 570 575
 Arg Glu Cys Ser Lys Leu Pro Leu Arg Ile Thr Tyr Tyr His Leu Ser
 580 585 590
 Phe Pro Thr Asn Ile Gln Ala Pro Ala Val Val Phe Arg Met Gly Pro
 595 600 605

-54-

Ser Ser Ala Val Pro Gly Asp Ser Met Gln Leu Ala Ile Thr Gly Gly
 610 615 620

Asn Glu Glu Gly Phe Phe Thr Thr Arg Lys Val Ser Pro His Ser Gly
 625 630 635 640

Val Val Ala Leu Thr Lys Pro Val Pro Glu Pro Arg Asp Leu Leu Leu
 645 650 655

Thr Val Lys Met Asp Leu Ser Arg His Gly Thr Val Ser Ser Phe Val
 660 665 670

Ala Lys Leu Phe Ile Phe Val Ser Ala Glu Leu
 675 680

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 703 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

- (ix) FEATURE: LEADER SEQUENCE=POSITIONS 1-29. SEE FIGURE 10 AND CORRESPONDING DESCRIPTION.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Met Glu Arg Ala Ala Pro Ser Arg Arg Val Pro Leu Pro Leu Leu Leu
 1 5 10 15

Leu Gly Gly Leu Ala Leu Leu Ala Ala Gly Val Asp Ala Asp Val Leu
 20 25 30

Leu Glu Ala Cys Cys Ala Asp Gly His Arg Met Ala Thr His Gln Lys
 35 40 45

Asp Cys Ser Leu Pro Tyr Ala Thr Glu Ser Lys Glu Cys Arg Met Val
 50 55 60

Gln Glu Gln Cys Cys His Ser Gln Leu Glu Glu Leu His Cys Ala Thr
 65 70 75 80

Gly Ile Ser Leu Ala Asn Glu Gln Asp Arg Cys Ala Thr Pro His Gly
 85 90 95

Asp Asn Ala Ser Leu Glu Ala Thr Phe Val Lys Arg Cys Cys His Cys
 100 105 110

Cys Leu Leu Gly Arg Ala Ala Gln Ala Gln Gly Gln Ser Cys Glu Tyr
 115 120 125

Ser Leu Met Val Gly Tyr Gln Cys Gly Gln Val Phe Arg Ala Cys Cys
 130 135 140

-55-

Val Lys Ser Gln Glu Thr Gly Asp Leu Asp Val Gly Gly Leu Gln Glu
 145 150 155 160
 Thr Asp Lys Ile Ile Glu Val Glu Glu Glu Gln Glu Asp Pro Tyr Leu
 165 170 175
 Asn Asp Arg Cys Arg Gly Gly Gly Pro Cys Lys Gln Gln Cys Arg Asp
 180 185 190
 Thr Gly Asp Glu Val Val Cys Ser Cys Phe Val Gly Tyr Gln Leu Leu
 195 200 205
 Ser Asp Gly Val Ser Cys Glu Asp Val Asn Glu Cys Ile Thr Gly Ser
 210 215 220
 His Ser Cys Arg Leu Gly Glu Ser Cys Ile Asn Thr Val Gly Ser Phe
 225 230 235 240
 Arg Cys Gln Arg Asp Ser Ser Cys Gly Thr Gly Tyr Glu Leu Thr Glu
 245 250 255
 Asp Asn Ser Cys Lys Asp Ile Asp Glu Cys Glu Ser Gly Ile His Asn
 260 265 270
 Cys Leu Pro Asp Phe Ile Cys Gln Asn Thr Leu Gly Ser Phe Arg Cys
 275 280 285
 Arg Pro Lys Leu Gln Cys Lys Ser Gly Phe Ile Gln Asp Ala Leu Gly
 290 295 300
 Asn Cys Ile Asp Ile Asn Glu Cys Leu Ser Ile Ser Ala Pro Cys Pro
 305 310 315 320
 Ile Gly His Thr Cys Ile Asn Thr Glu Gly Ser Tyr Thr Cys Gln Lys
 325 330 335
 Asn Val Pro Asn Cys Gly Arg Gly Tyr His Leu Asn Glu Glu Gly Thr
 340 345 350
 Arg Cys Val Asp Val Asp Glu Cys Ala Pro Pro Ala Glu Pro Cys Gly
 355 360 365
 Lys Gly His Arg Cys Val Asn Ser Pro Gly Ser Phe Arg Cys Glu Cys
 370 375 380
 Lys Thr Gly Tyr Tyr Phe Asp Gly Ile Ser Arg Met Cys Val Asp Val
 385 390 395 400
 Asn Glu Cys Gln Arg Tyr Pro Gly Arg Leu Cys Gly His Lys Cys Glu
 405 410 415
 Asn Thr Leu Gly Ser Tyr Leu Cys Ser Cys Ser Val Gly Phe Arg Leu
 420 425 430
 Ser Val Asp Gly Arg Ser Cys Glu Asp Ile Asn Glu Cys Ser Ser Ser
 435 440 445

-56-

Pro Cys Ser Gln Glu Cys Ala Asn Val Tyr Gly Ser Tyr Gln Cys Tyr
 450 455 460
 Cys Arg Arg Gly Tyr Gln Leu Ser Asp Val Asp Gly Val Thr Cys Glu
 465 470 475 480
 Asp Ile Asp Glu Cys Ala Leu Pro Thr Gly Gly His Ile Cys Ser Tyr
 485 490 495
 Arg Cys Ile Asn Ile Pro Gly Ser Phe Gln Cys Ser Cys Pro Ser Ser
 500 505 510
 Gly Tyr Arg Leu Ala Pro Asn Gly Arg Asn Cys Gln Asp Ile Asp Glu
 515 520 525
 Cys Val Thr Gly Ile His Asn Cys Ser Ile Asn Glu Thr Cys Phe Asn
 530 535 540
 Ile Gln Gly Ala Phe Arg Cys Leu Ala Phe Glu Cys Pro Glu Asn Tyr
 545 550 555 560
 Arg Arg Ser Ala Ala Thr Leu Gln Gln Glu Lys Thr Asp Thr Val Arg
 565 570 575
 Cys Ile Lys Ser Cys Arg Pro Asn Asp Val Thr Cys Val Phe Asp Pro
 580 585 590
 Val His Thr Ile Ser His Thr Val Ile Ser Leu Pro Thr Phe Arg Glu
 595 600 605
 Phe Thr Arg Pro Glu Glu Ile Ile Phe Leu Arg Ala Ile Thr Pro Pro
 610 615 620
 His Pro Ala Ser Gln Ala Asn Ile Ile Phe Asp Ile Thr Glu Gly Asn
 625 630 635 640
 Leu Arg Asp Ser Phe Asp Ile Ile Lys Arg Tyr Met Asp Gly Met Thr
 645 650 655
 Val Gly Val Val Arg Gln Val Arg Pro Ile Val Gly Pro Phe His Ala
 660 665 670
 Val Leu Lys Leu Glu Met Asn Tyr Val Val Gly Gly Val Val Ser His
 675 680 685
 Arg Asn Val Val Asn Val Arg Ile Phe Val Ser Glu Tyr Trp Phe
 690 695 700

CLAIMS:

1. A pharmaceutical composition consisting of fibulin protein in a pharmaceutically acceptable
5 diluent, adjuvant or carrier, said fibulin protein being selected from among:
- (a) the amino acid sequence 1 - 537 as set forth in SEQ. ID. NO. 7;
 - (b) the amino acid sequence 1 - 572 as
10 set forth in SEQ. ID. NO. 8;
 - (c) the amino acid sequence 1 - 654 as set forth in SEQ. ID. NO. 9;
 - (d) the amino acid sequence 1 - 672 as set forth in SEQ. ID. NO. 10;
 - 15 (e) the amino acid sequence of any of subparts (a), (b), (c), or (d) having a methionyl residue at the N terminus;
 - (f) the amino acid sequence of any of subparts (a), (b), (c), or (d) having a different amino
20 acid substituted in one or more of the following positions:
 - (i) one or more cysteine residues replaced by an alanine or serine residue;
 - (ii) one or more tyrosine residues
25 replaced by a phenylalanine residue;
 - (g) a truncated fibulin protein analog sufficient to bind an OB protein, analog or derivative thereof;
 - (h) a hybrid molecule of fibulin protein
30 selected from among the following:
 - (i) amino acids 1-537 and
(connected to) amino acids 573-654 or amino acids 655-674;
 - (ii) amino acids 1-572 and
35 (connected to) amino acids 655-674;

(iii) amino acids 1-537 and
(connected to) amino acids 573-654, having one or
more of amino acids 538-572 placed between amino
acids 537 and 573;

5 (iv) amino acids 1-537 and
(connected to) amino acids 655-674, having one or
more of amino acids 538-572 and 573-654, placed
between amino acids 537 and 655; and

(v) amino acids 1-572 and (connected
10 to) amino acids 655-674, having one or more of
amino acids 573-654 placed between amino acids 572
and 655;

(i) the fibulin protein, analog or derivative
of any of subparts (a) through (h) comprised of a
15 chemical moiety connected to the protein moiety;

(j) a derivative of subpart (i) wherein said
chemical moiety is a water soluble polymer moiety;

(k) a derivative of subpart (j) wherein said
water soluble polymer moiety is polyethylene glycol;

20 (l) a derivative of subpart (j) wherein said
water soluble polymer moiety is a polyamino acid moiety;

(m) a derivative of subpart (j) wherein said
water soluble polymer moiety is attached at solely the
N-terminus of said protein moiety;

25 (n) a combination of fibulin protein from any
of subparts (a) through (m); and

(o) a dimer fibulin protein from any of
subparts (a) through (m).

2. A pharmaceutical composition of Claim 1
30 comprising said fibulin protein of subpart (a).

3. A pharmaceutical composition of Claim 1
comprising said fibulin protein of subpart (b).

4. A pharmaceutical composition of Claim 1 comprising said fibulin protein of subpart (c).

5. A pharmaceutical composition of Claim 1 comprising said fibulin protein of subpart (d).

6. A pharmaceutical composition of Claim 1 comprising said fibulin protein of subpart (e).

7. A complex consisting of a fibulin molecule selected from among fibulin type 1 variants A, B, C, D (SEQ. ID. NOS. 7, 8, 9, and 10), analogs or derivatives thereof, complexed to an OB protein, analog or derivative thereof selected from among:

(a) the amino acid sequence 1-146 as set forth in SEQ. ID. NO. 3 or SEQ ID. NO. 6 ;

(b) the amino acid sequence 1-146 as set forth in SEQ. ID. NO. 6 having a lysine residue at position 35 and an isoleucine residue at position 74;

(c) the amino acid sequence of subpart (b) having a different amino acid substituted in one or more of the following positions (using the numbering according to SEQ. ID. NO. 6): 4, 8, 32, 33, 35, 48, 50, 53, 60, 64, 66, 67, 68, 71, 74, 77, 78, 89, 97, 100, 102, 105, 106, 107, 108, 111, 112, 118, 136, 138, 142, and 145;

(d) the amino acid sequence of subparts (a), (b) or (c) optionally lacking a glutaminy residue at position 28;

(e) the amino acid sequence of subparts (a), (b), (c), or (d) having a methionyl residue at the N terminus.

(f) a truncated OB protein analog (using the numbering of SEQ. ID. NO. 6 having a lysine residue

at position 35, and an isoleucine residue at position 74) selected from among:

- 5 (i) amino acids 98-146;
(ii) amino acids 1-32;
(iii) amino acids 40-116;
(iv) amino acids 1-99 and 112-146;
(v) amino acids 1-99 and 112-146
having one or more of amino acids 100-111
sequentially placed between amino acids 99 and 112;
- 10 (vi) the truncated OB analog of
subpart (f)(i) having one or more of amino acids
100, 102, 105, 106, 107, 108, 111, 112, 118, 136,
138, 142, and 145 substituted with another amino
acid;
- 15 (vii) the truncated analog of
subpart (f)(ii) having one or more of amino acids
4, 8 and 32 substituted with another amino acid;
- (viii) the truncated analog of
subpart (f)(iii) having one or more of amino acids
20 50, 53, 60, 64, 66, 67, 68, 71, 74, 77, 78, 89,
97, 100, 102, 105, 106, 107, 108, 111 and 112
replaced with another amino acid;
- (vix) the truncated analog of
subpart (f)(iv) having one or more of amino acids
25 4, 8, 32, 33, 35, 48, 50, 53, 60, 64, 66, 67, 68,
71, 74, 77, 78, 89, 97, 112, 118, 136, 138, 142,
and 145 replaced with another amino acid;
- (x) the truncated analog of subpart
(f)(v) having one or more of amino acids 4, 8, 32,
30 33, 35, 48, 50, 53, 60, 64, 66, 67, 68, 71, 74,
77, 78, 89, 97, 100, 102, 105, 106, 107, 108, 111,
112, 118, 136, 138, 142, and 145 replaced with
another amino acid;

(xi) the truncated analog of any of subparts (f)(i)-(x) having an N-terminal methionyl residue; and

(g) the OB protein or analog derivative
5 of any of subparts (a) through (f) comprised of a chemical moiety connected to the protein moiety;

(h) a derivative of subpart (g) wherein said chemical moiety is a water soluble polymer moiety;

(i) a derivative of subpart (h) wherein
10 said water soluble polymer moiety is polyethylene glycol;

(j) A derivative of subpart (h) wherein said water soluble polymer moiety is a polyamino acid moiety; and

15 (k) a derivative of subpart (h) wherein said water soluble polymer moiety is attached at solely the N-terminus of said protein moiety.

8. A complex of Claim 7 comprising said OB
20 protein of subpart (a).

9. A complex of Claim 7 comprising said OB protein of subpart (b).

25 10. A pharmaceutical composition consisting of a complex according to Claim 7 or combination of complexes thereof in a pharmaceutically acceptable diluent, adjuvant or carrier.

30 11. A pharmaceutical composition of Claim 10 wherein said complex comprises said OB protein of subpart (a).

12. A pharmaceutical composition of Claim 10 wherein said complex comprises said OB protein of subpart (b).

5 13. A method of making a pharmaceutical composition by admixing purified and isolated fibulin protein according to Claim 1 or combination thereof, with a pharmaceutically acceptable diluent, adjuvant, or carrier.

10

 14. A method of making a pharmaceutical composition by admixing purified and isolated complex according to Claim 7 or combination thereof, with a pharmaceutically acceptable diluent, adjuvant, or
15 carrier.

 15. A method of treatment of a disorder selected from among excess weight, high blood lipid levels, and type II diabetes, said method consisting of
20 administering a therapeutically effective amount of Fibulin type 1 variants A, B, C, or D or analogs, derivatives or combinations thereof.

 16. A method of treatment of a disorder
25 selected from among excess weight, high blood lipid levels, and type II diabetes, said method consisting of administering a therapeutically effective amount of a complex according to Claim 7 or combination of complexes thereof.

30

 17. A method of increasing lean tissue mass, said method consisting of administering a therapeutically effective amount of fibulin type 1 protein, including variants A, B, C, or D or analogs,
35 derivatives or combinations thereof.

18. A method of increasing lean tissue mass,
said method consisting of administering a
therapeutically effective amount of a complex according
5 to Claim 7 or combination of complexes thereof.

19. A method for detecting the presence of OB
protein in a biological sample comprising:

incubating the sample with fibulin protein
10 according to Claim 1, or combination thereof, under
conditions that allow binding of the fibulin protein to
OB protein to form a complex; and

measuring the amount of OB protein in the
bound complex.

15

1 / 9

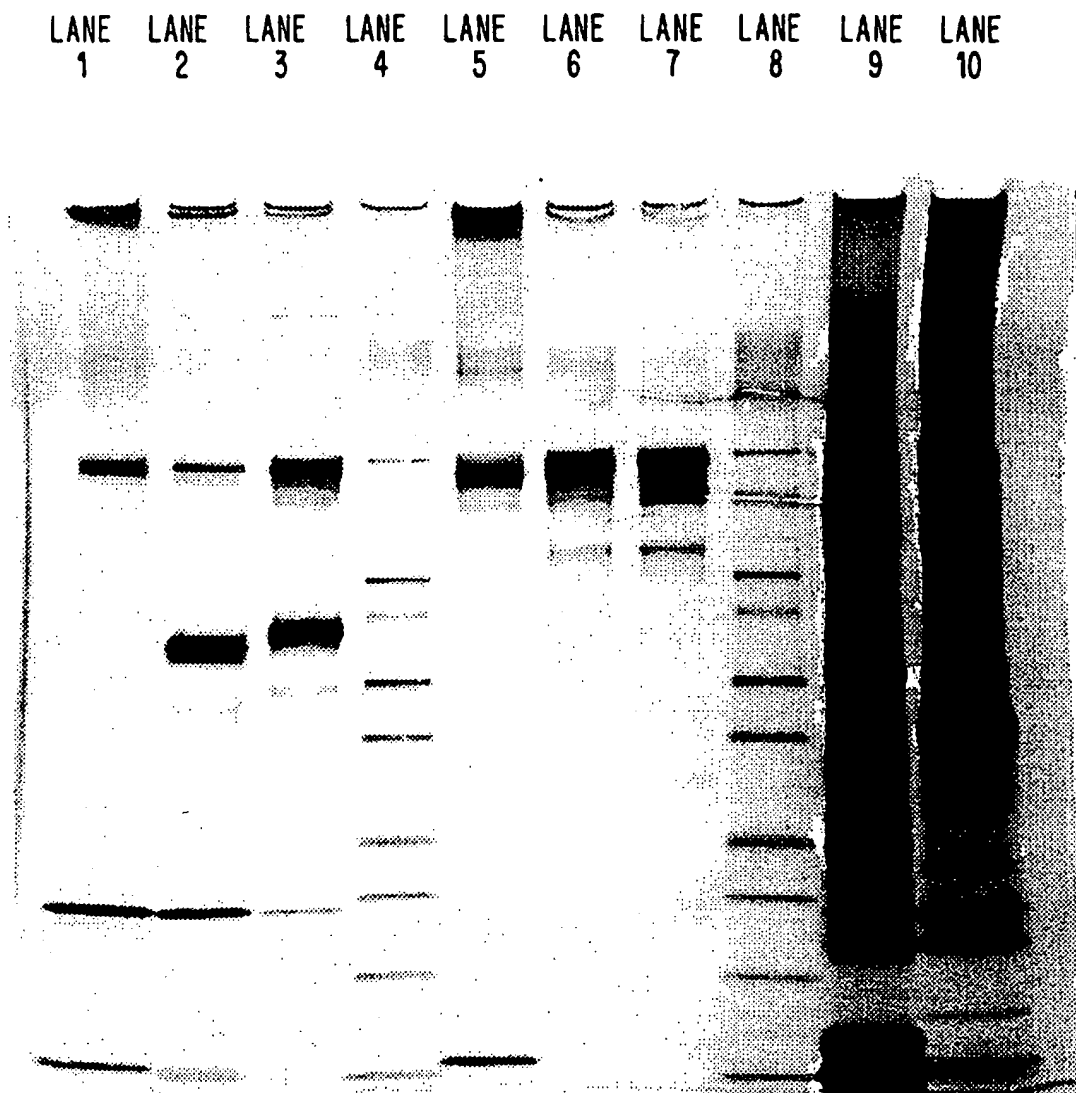


FIG. 1

FIG. 2A

9 TCTAGATTGAGTTTAACTTTTAGAAGGAGGAATAACATATGGTACCGATCCAGAAAGT 68
 -+-----+-----+-----+-----+-----+-----+-----+-----
 AGATCTAAACTCAAAATGAAAATCTCCCTCTATTGTATACCATGGCTAGGTCTTTCA
 M V P I Q K V -
 69 TCAGGACGACACCAACCTTAATTAAACGATCGTTACGCGTATCAACGACATCAGTCA 128
 -+-----+-----+-----+-----+-----+-----+-----+-----
 AGTCCTGCTGTGGTTTGGAAATTAATTTGCTAGCAATGCCGATAGTTGCTGTAGTCAGT
 Q D T K T L I K T I V T R I N D I S H -
 129 CACCCAGTCGGTCTCCGCTAAACAGCGTGTTACCGGTCTGGACTTCATCCCGGGTCTGCA 188
 -+-----+-----+-----+-----+-----+-----+-----+-----
 GTGGGTCAGCCAGAGGCGATTGTGCGACAAATGGCCAGACCTGAAGTAGGGCCCCAGACGT
 T Q S V S A K Q R V T G L D F I P G L H -
 189 CCCGATCCTAAGCTTGTCAAAATGGACCAGACCCCTGGCTGTATACCAGCAGGTGTTAAC 248
 -+-----+-----+-----+-----+-----+-----+-----+-----
 GGGCTAGGATTCGAACAGGTTTACCTGGTCTGGGACCGACATATGGTCGTCACCAATTG
 P I L S L S K M D Q T L A V Y Q Q V L T -

FIG. 2B

CTCCCTGCCGTCCCAGAACGTTCTTCAGATCGCTAACGACCTCGAGAACCTTCGCACCT
-+-----+-----+-----+-----+-----+-----+-----+
249 GAGGGACGGCAGGCTCTGCAAGAAGTCTAGCGATTGCTGGAGCTCTTGGAAGCGCTGGA
S L P S Q N V L Q I A N D L E N L R D L -
-
GCTGCACCTGCTGGCATCTCCAATACTCTGCTCCCTGCCGCAGACCTCAGGTCTTCAGAA
-+-----+-----+-----+-----+-----+-----+-----+
309 CGACGTGGACACCGTAAGAGGTTTAGGACGAGGACGGCGTCTGGAGTCCAGAAGTCTT
L H L L A F S K S C S L P Q T S G L Q K -
-
ACCGGAATCCCTGGACGGGTCTTGGAAGCATCCCTGTACAGCACCGAAGTTGTGCTCT
-+-----+-----+-----+-----+-----+-----+-----+
369 TGGCCTTAGGGACCTGCCCCAGGACCCTTCGTAGGGACATGTCGTGGCTTCAACAACGAGA
P E S L D G V L E A S L Y S T E V V A L -
-
GTCCCCGTCTGCAGGGTTCCCTTCAGGACATCCCTTCAGCAGCTGGACGTTTCTCCGGAATG
-+-----+-----+-----+-----+-----+-----+-----+
429 CAGGGCAGACGTCCC AAGGGAAGTCCCTGTAGGAAGTCGTGACCTGCAAAGAGGCCTTAC
S R L Q G S L Q D I L Q Q L D V S P E C -
-
TTAATGGATCC
-+-----+-----+-----+-----+-----+-----+-----+
489 AATTACCTAGG

FIG. 3A

1 CATATGGTACCGATCCAGAAAGTTCAGGACGACACCAAAACCTTAATTAACGATCGTT 60
 -----+-----+-----+-----+-----+-----+
 GTATACCATGGCTAGGCTCTTCAAGTCCTGCTGGTTTGGAAATTAATTTGCTAGCAA
 M V P I Q K V Q D D T K T L I K T I V -
 61 ACGCGTATCAACGACATCAGTCACACCCAGTCGGTGAGCTCTAAACAGCGTGTACAGGC 120
 -----+-----+-----+-----+-----+-----+
 TGCGCATAGTTGCTGTAGTCAGTGTGGGTCAGCCACTCGAGATTTGTCGCACAATGTCCG
 T R I N D I S H T Q S V S S K Q R V T G -
 121 CTGGACTTCATCCCGGGTCTGCACCCGATCCCTGACCTTGTCAAAATGGACACCCCTG 180
 -----+-----+-----+-----+-----+-----+
 GACCTGAAGTAGGGCCCCAGACCGTGGGCTAGGACTGGAACAGGTTTACCTGGTCTGGGAC
 L D F I P G L H P I L T L S K M D Q T L -
 181 GCTGTATACCAGCAGATCTTAACCTCCATGCCGTCCCGTAACGTTCTTCAGATCTCTAAC 240
 -----+-----+-----+-----+-----+-----+
 CGACATATGGTCGTCTAGAATTGGAGGTACGGCAGGGCATTGCAAGAAGTCTAGAGATTG
 A V Y Q Q I L T S M P S R N V L Q I S N -

FIG.3B

241 -----+-----+-----+-----+-----+-----+-----+
 GACCTCGAGAACCTTCGCGACCTGCTGCACGTGCTGGCATCTCCAATCCTGCCACCTG
 -----+-----+-----+-----+-----+-----+-----+ 300
 CTGGAGCTCTTGGAAGCGCTGGACGACGTGCACGACCGTAAGAGGTTAGGACGGTGGAC
 D L E N L R D L L H V L A F S K S C H L -
 301 -----+-----+-----+-----+-----+-----+-----+
 CCATGGGCTTCAGGTCTTGAGACTCTGGAACCTCTGCGCGGGTCTCTGGAAGCATCCGGT
 -----+-----+-----+-----+-----+-----+-----+ 360
 GGTACCCGAAGTCCAGAACTCTGAGACCTGAGAGACCCGCCAGGACCTTCGTAGGCCA
 P W A S G L E T L D S L G G V L E A S G -
 361 -----+-----+-----+-----+-----+-----+-----+
 TACAGCACCGAAGTTGTTGCTCTGTCCCGTCTGCAGGGTTCCCTTCAGGACATGCTTTGG
 -----+-----+-----+-----+-----+-----+-----+ 420
 ATGTCGTGGCTTCAACAACGAGACAGGGCAGACGTCCCAAGGGAAGTCCTGTACGAAACC
 Y S T E V V A L S R L Q G S L Q D M L W -
 421 -----+-----+-----+-----+-----+-----+-----+
 CAGCTGGACCTGTCTCCGGGTTGTTAATGGATCC
 -----+-----+-----+-----+-----+-----+-----+ 454
 GTCGACCTGGACAGAGGCCCAACAATTACCTAGG
 Q L D L S P G C *

5 / 9

FIG.4

1
-29 MERAAPSRV PLPLLLGL ALLAAGVDAD VLEACCADG HRMATHQKDC
22 SLPYATESKE CRMVQEQCCH SQLEELHCAT CATPHGDNAS
77 LEATFVKRCC HCCLLGRAAQ AQQQSCEYSL MVGYQCGQVF RACCVKSQET
122 GDLDVGGLQE TDKIIEVEEE QEDPYLNDRC RGGGPKQQC RDTGDEVVCS
172 CFVGYQLLSD GVSCEDVNEC ITGSHSCLRG ESCINTVGSF RCQRDSSCGT
222 GYELTEDNSC KDIDECESGI HNCLPDFICQ NTLSFRCRP KLQCKSGFIQ
272 DALGNCIDIN ECLSIAPCP IGHTCINTEG SYTCQKNVPN CGRGYHLNEE
322 GTRCVDVDEC APPAEPCKG HRCVNSPGSF RCECKTGYF DGISRMCVDV
372 NECQRYPGRL CGHKCENTLG SYLSCSVGF RLSVDGRSCE DINECSSPC
422 SQECANVYGS YQCYCRRGYQ LSDVDGVTC DIDEALPTG GHICSYRCIN
472 IPGSEQCSCP SSGYRLAPNG RNCQIDECV TGIHNC SINE TCFNIQGAFR
522 CLAFECPENY RRSAAAT

7 / 9

FIG.5

1
-29 MERAAPSRV PLPLLLGGL ALLAAGVDAD VLLEACCADG HRMATHQKDC
22 SLPYATESKE CRMVQEQQCH SQLEELHCAT GISLANEQDR CATPHGDNAS
72 LEATFVKRCC HCCLLGRAAQ AQQQSCEYSL MVGYQCGQVF RACCVKSQET
122 GDLDVGGLQE TDKIIEVEEE QEDPYLNDRC RGGGPCQQC RDTGDEVVCS
172 CFVGYQLLSD GVSCEDVNEC ITGSHSCRLG ESCINTVGSF RCQRDSSCGT
222 GYELTEDNSC KDIDECESGI HNCPLDFICQ NTLGSFRCRP KLQCKSGFIQ
272 DALGNCIDIN ECLISISAPCP IGHTCINTEG SYTCQKNVNP CGRGYHLNEE
322 GTRCVDVDEC APPAEPCKG HRCVNSPGSF RCECKTGYF DGISRMCVDV
372 NECQRYPGRL CGHKCENTLG SYLCSCSVGF RLSVDGRSCE DINECSSPC
422 SQECANVYGS YQCYCRRGYQ LSDVDGVTCE DIDEALPTG GHICSYRCIN
472 IPGSFQCSCP SSGYRLAPNG RNCQDIDECV TGIHNCSINE TCFNIQGAFR
522 CLAFECPENY RRSAAATQSK KGRQNTPAGS SKEDCRVLPW KQGLDTHLD
572 A

FIG. 6

1
-29 MERAAPSRV PLPLLLGLL ALLAAGVDAD VLLEACCADG HRMATHQKDC
22 SLPYATESKE CRMVQEQCCH SQLEELHCAT GISLANEQDR CATPHGDNAS
72 LEATFVKRCC HCCLLGRAAQ AQQSCEYSL MVGYQCGQVF RACCVKSQET
122 GDLDVGGLQE TDKIIEVEEE QEDPYLNDRC RGGGPKKQQC RDTGDEVVCS
172 CFVGYQLLSD GVSCEDVNEC ITGSHSCLRG ESCINTVGSF RCQRDSSCGT
222 GYELTEDNSC KDIDECESGI HNCLPDFICQ NTLGSFRCRP KLQCKSGFIQ
272 DALGNCIDIN ECLSISAPCP IGHTCINTEG SYTCQKNVNP CGRGYHLNEE
322 GTRCVDVDEC APPAEPCKG HRCVNSPGSF RCECKTGYYF DGISRMCDV
372 NECQRYPGRL CGHKCENTLG SYLSCSVGF RLSVDGRSCE DINECSSSPC
422 SQECANVYGS YQCYCRRGYQ LSDVDGVTCE DIDEALPTG GHICSYRCIN
472 IPGSFQCSCP SSGYRLAPNG RNCQDIDECV TGIHNCSINE TCFNIQGAER
522 CLAFECPENY RRSAAATRCR LPCHENRECS KLPLRITYYH LSFPTNIQAP
572 AVVFRMGPS AVPGDSMQLA ITGGNEEGFF TTRKVSPHSG VVALTKPVPE
622 PRDLLLTVKM DLSRHGTVSS FVAKLFIQVS AEL

FIG. 7

1

-29 MERAAPSRV PLPLLLGLL ALLAAGVDAD VLEACCADG HRMATHQKDC
22 SLPYATESKE CRMVQECCCH SQLEELHCAT GISLANEQDR CATPHGDNAS
72 LEATFVKRCC HCCLLGRAAQ AQQSCSEYSL MVGYYCQQVF RACCVKSQET
122 GDLDVGGLQE TDKIIEVEEE QEDPYLNDRC RGGGPCKQQC RDTGDEVVCS
172 CFVGYQLLSD GVSCEDVNEC ITGSHSCRLG ESCINTVGSF RCQRDSSCGT
222 GYELTEDNSC KDIDECESGI HNCLPDFICQ NTLGSFRCRP KLQCKSGFIQ
272 DALGNCIDIN ECLSISAPCP IGHTCINTEG SYTCQKNVPN CGRGYHLNEE
322 GTRCVDVDEC APPAEPCKG HRCVNSPGSF RCECKTGYF DGISRMCVDV
372 NECQRYPGRL CGHKCENTLG SYLCSCSVGF RLSVDGRSCE DINECSSSPC
422 SQECANVYGS YQCYCRRGYQ LSDVDGVTCE DIDEALPTG GHICSYRCIN
472 IPGSFQCSCP SSGYRLAPNG RNCQDIDECV TGIHNCSINE TCFNIQGAFR
522 CLAFECPENY RRSAAATLQOE KTDTVRCIKS CRPNDVTCVF DPVHTISHTV
572 ISLPTFREFT RPEEIIFLRA ITPPHPASQA NIIFDITEGN LRDSFDIIKR
622 YMDGMTVGUV RQVRPIVGPF HAVLKLEMNY VVGGVVSHRN VVNVRIEVS
672 YWF

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/06280

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07K14/575 C07K14/78 A61K38/22 A61K38/39 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 91 02755 A (LA JOLLA CANCER RESEARCH FOUNDATION ET AL.) 7 March 1991 cited in the application see the whole document ---	1,2
X	CELL, vol. 58, 25 August 1989, NA US, pages 623-629, XP002036653 W S ARGRAVES ET AL.: "Fibulin, a novel protein that interacts with the fibronectin receptor beta subunit cytoplasmic domain" cited in the application see the whole document --- -/-	1,2

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *Z* document member of the same patent family

Date of the actual completion of the international search

31 July 1997

Date of mailing of the international search report

18.08.97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+ 31-70) 340-3016

Authorized officer

Masturzo, P

INTERNATIONAL SEARCH REPORT

In. Application No

PCT/US 97/06280

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEMICAL ABSTRACTS, vol. 114, no. 7, 18 February 1991 Columbus, Ohio, US; abstract no. 57681, XP002036654 see abstract & J CELL BIOL., vol. 111, no. 6, pt. 2, 1989, pages 3155-3164, W S ARGRAVES ET AL.: "Fibulin is an extracellular matrix and plasma glycoprotein with repeated domain structures" cited in the application ---	1-6
A	SCIENCE, vol. 269, 28 July 1995, LANCASTER, PA US, pages 543-546, XP000602064 J L HALAAS ET AL.: "Weight-reducing effects of the plasma" cited in the application see the whole document -----	7-19

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 97/06280

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 15-18
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 15 to 18 refer to a method of treatment of the human body, the search has been carried out and based on the alleged effects of the products.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

In International Application No

PCT/US 97/06280

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9102755 A	07-03-91	AU 6299390 A	03-04-91
		CA 2064818 A	19-02-91
		EP 0484452 A	13-05-92
		JP 5503288 T	03-06-93
